BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

REGULATION OF COLα1(I), LO AND COX-1 mRNA EXPRESSION BY PROSTAGLANDIN E_2 IN HUMAN LUNG EMBRYONIC FIBROBLASTS, IMR-90

by

JAMES K. CHOUNG

B.S., United States Military Academy, 1989

Submitted in partial fulfillment of the

Requirements for the degree of

Master of Arts

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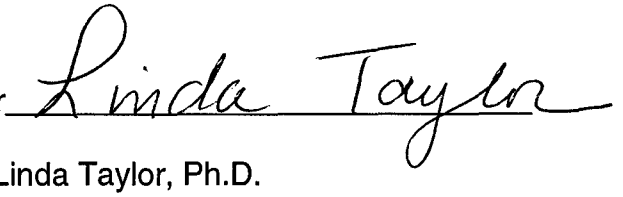
First Reader 

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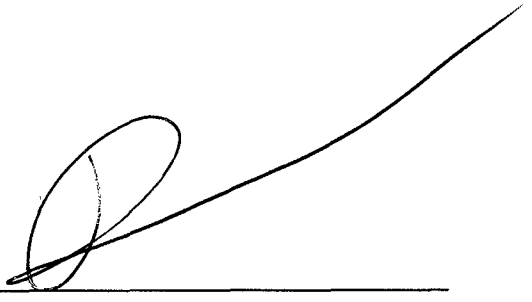
Second Reader 

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REGULATION OF COLα1(I), LO AND COX-1 mRNA EXPRESSION BY PROSTAGLANDIN E_2 IN HUMAN LUNG EMBRYONIC FIBROBLASTS, IMR-90

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ABSTRACT

PGE_2 decreases the synthesis of collagen and lysyl oxidase, key factors involved in fibrosis and tissue scarring. The mechanism by which PGE_2 induces its inhibitory effect has been explored in human embryonic lung fibroblasts, IMR-90, and Rat-1 cells. In IMR-90, PGE_2 limited COLα1(I) and LO mRNA expressions, but increased mRNA level of COX-1 which catalyzes PGE_2 synthesis. Through Northern blot analysis, EP2, EP3 and EP4 receptor mRNAs were detected. Among these, EP2 mRNA expression was predominant, while EP3 and EP4 mRNAs were expressed in lesser degree with EP3 expression showing the least amount. EP1 receptor was not detected. Agonists, 11-deoxy PGE_1 for EP2/EP4 and sulprostone for EP3/EP1, were used to determine the receptor type and possible signaling mechanism involved in mediating the PGE_2 effect. 11-deoxy PGE_1 demonstrated similar potency in limiting not only the basal but also TGF-β and IL-1β stimulated COLα1(I) and LO mRNA levels, respectively. Sulprostone showed no effect. Since EP2 and EP4 are known to generate cAMP, forskolin

was used to examine this possibility. Forskolin showed similar results as PGE_2 and 11-deoxy PGE_1. However, forskolin with PGE_2 showed greater inhibition of COLα1(I) mRNA level than either effector, alone. Rat-1 cells were used to further examine the importance of EP2 receptor. In contrast to IMR-90, PGE_2 had no effect on COLα1(I) mRNA level, but forskolin increased it by almost 10-fold. Interestingly, only EP4 mRNA was detected in Rat-1 cells. These results suggest that EP2 is crucial in suppressing COLα1(I) and LO mRNA levels, subsequently limiting collagen deposition.

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INTRODUCTION

Overview

Deposition of large amounts of collagen is one of the prominent attributes of fibrosis [Zhang et al., 1994; McDonald et al., 1986] and tissue scarring [Whitby and Ferguson, 1991]. The regulation of collagen deposition involves a number of effectors, including prostaglandin E_2 (PGE_2) [Goldstein and Polgar, 1982; Raisz et al., 1993], an enzyme, lysyl oxidase (LO) [Siegel et al., 1970; Rucker and Murray, 1978], and cytokines such as transforming growth factor-beta (TGF-β) [Fine et al., 1990; Diaz et al., 1989; Krupsky et al., 1996] and interleukin-1 (IL-1) [Bhatnagar et al., 1986; Kahari et al., 1987; Pasternak et al., 1986; El Maradny et al., 1996]. The effect of PGE_2 on the regulation of the type I collagen α1 [COLα1(I)] synthesis and deposition at the receptor and transcriptional levels is the focus of this dissertation.

In response to tissue injury, macrophages release a plethora of membrane-perturbing stimuli including IL-1β [Cannon et al., 1992, Zhang et al., 1993] and TGF-β in wound cleft [Broadly et al., 1988; Grotendorst et al., 1988]. One of the initial effects of these cytokines seems to result in an overall increase in collagen synthesis. TGF-β is widely known to cause an increase in collagen synthesis by cells such as fibroblasts [Reed et al., 1994; Gallivan et al., 1997; Krupsky et al., 1996; Fine et al., 1990; Diaz et al., 1989]. On the other hand, IL-1β has been found to increase the synthesis of LO [Roy et al., 1996] which is an active participant during collagen deposition [Pinnell and Martin, 1968]. In

addition, these two cytokines actively induce PGE_2 synthesis, apparently through arachidonate metabolism catalyzed by cyclooxygenase-1 (COX-1) and COX-2 [Jackson et al., 1993]. Of the many responses PGE_2 elicits during the immune response, it decreases collagen levels, both steady state [Brilla et al., 1995; Goldstein and Polgar, 1982; Fine et al., 1992] and TGF-β stimulated levels [Fine et al., 1990; Diaz et al., 1989; Krupsky et al., 1996]. Also, it decreases both steady state and IL-1β stimulated LO mRNA levels [Roy et al., 1996].

This evidence suggests that PGE_2 and its receptor(s) are important factors, if not the central factors, in collagen deposition. PGE_2 induces its effect in an autocrine fashion through four receptor subtypes, EP1, EP2, EP3 and EP4 [Coleman et al., 1994; Ichikawa et al., 1996]. It is not known which EP receptor subtype(s) is/are involved in effecting collagen deposition. The first objective of this research is to determine the EP receptor subtype profile in two *in vitro* models, mortal human embryonic lung fibroblasts (IMR90) and immortal rat embryonic fibroblasts (Rat-1). The second objective is to pinpoint the receptor subtype(s) involved in mediating the PGE_2 effect on collagen deposition. The third objective is to determine the secondary signal messenger involved in the receptor subtype(s) signal transduction. By achieving these objectives, the investigator hopes to provide some insight into critical aspects of collagen deposition that may contribute to therapeutic ramifications in fibrosis and tissue scarring.

Collagen and Lysyl Oxidase

Fibrosis develops from an excessive accumulation of collagen in the extracellular matrix and demonstrates many characteristics of wound healing following tissue injury [Siegel, 1979]. In fibrotic lung, such as in idiopathic chronic fibrosis, the increase in the total amount of the type I collagen synthesis and its deposition in the interstitium and alveolar spaces are the most prominent features [Seyer et al., 1976].

Collagen molecules consist of three helical polypeptides, called α chains, wound about each other, forming a triple helix. Type I collagen [Birk and Silver, 1984] usually consists two α1(I) chains and one α2(I) chain [Piez et al., 1963], or homotrimers of α1(I) chains, if no α2(I) is produced [Miller and Matukas, 1969]. Type I collagen is fibril forming collagen and is one of the main types found in the connective tissues [Bornstein, 1974]. It is not only the principal collagen of skin and bone, but also the major interstitial collagen [Bornstein, 1974].

The most commonly held view of collagen molecule folding begins with synthesis of procollagen from the three proα-chains [Bornstein, 1974; Bienkowski and Gotkin, 1995]. The proα-chains are rich in proline and glycine which appear to play important roles in triple helix formation. Each chain consists of about 1000 amino acid residues [Kang et al., 1967]. The chain is arranged in a left-handed helix with three amino acid residues per turn and contains glycine as every third residue [Bornstein, 1974]. Its sequence is widely viewed as Gly-X-Y, where X is commonly proline and Y is proline or hydroxyproline. According to

Bornstein (1974), the synthesis of a proα-chain begins on the membrane-bound ribosomes, and it is injected into the lumen of the endoplasmic reticulum. The synthesized proα-chain becomes hydroxylated at selected proline and lysine residues with some glycosylation at selected hydroxylysine residues.

Subsequently, the three proα-chains wind about each other with extension peptides at the amino (N-) and carboxy (C-) termini, forming a triple helix. The helical formation in each chain is stabilized in part by the ring structure of proline, while glycine, with its small size, allows the tight packing of the three helical chains to form the ultimate triple helix. The triple helix is further stabilized with hydrogen bonds between the chains.

The triple helix is secreted into the extracellular matrix [Lenaers et al., 1971] as procollagen via secretory vesicles formed in the Golgi apparatus. The extension peptides of procollagen are then cleaved by the procollagen peptidases, producing collagen molecules [Lapiere et al., 1971]. The collagen molecules then associate laterally and head-to-end, ultimately forming fibrils [Robins and Duncan, 1983].

Thus far, almost 20 genetically distinct collagens have been found [Bienkowski and Gotkin, 1995] while about 25 α chains have been identified. The type I collagen accounts for 90% of body collagen and it is found in many tissues, including bone, skin, tendon, ligaments, cornea, and internal organs. The genes coding for the proα1(I) and proα2(I) chains, COL1A1 and COL1A2, are located on Chromosomes 17 and 7, respectively [Sandell and Boyd, 1990].

The tensile strength of collagen fibrils is achieved through covalent cross-links between lysine residues of collagen molecules within the fibril. Such cross-linking is initiated through LO in the extracellular space. LO is an amine oxidase which contains a copper ion and an aromatic carbonyl (pyridoxal 5'-phosphate or pyrroloquinoline quinone) as cofactors [Myers et al., 1985; Kagan, 1986]. Its molecular weight in various human tissues, determined through Western blotting of urea extracts, is approximately 30 kilo Daltons (kDa) [Kuivaniemi, 1985].

The LO oxidatively deaminates ε-amino groups of lysyl or hydroxylysyl residues in collagen fibrils to peptidyl α-aminoadipic-δ-semialdehyde [Pinnell and Martin, 1968]. This process yields highly reactive aldehyde groups, allysine or hydroxyallysine, respectively, which spontaneously condense with the other vicinal aldehydes to form aldol cross-links, or with the other ε-amino groups of lysyl or hydroxylysyl residues to form Schiff base cross-links [Siegel, 1979].

The intramolecular covalent bonds, or the cross-links, in the triple helix of proα-chains occur at two allysine residues located at the short non-helical, terminal region of the chains [Robins and Duncan, 1983]. These reactive residues combine in aldol-type condensation, known as allysine aldol. On the other hand, intermolecular cross-links results from one of the two pathways, one based on lysine aldehyde pathway and the other on hydroxylysine aldehyde pathway [Ricard-Blum and Ville, 1989]. These cross-links allow formation of covalent bonds between different α chains of the same collagen type or different

collagen types. The selection of the crosslinking pathway appears to be dictated by the type of tissue in which collagen is produced [Eyre et al., 1984].

To date, no one seems to repute the suggestion of Rucker and Murray (1978) that the enzymatic activity of LO may be the sole initiator of cross-link formation for collagen. As early as 1968, Peacock et al., had shown that inhibition of the cross-linking process with lathyrogens, an irreversible lysyl oxidase inhibitor, apparently limited scar formation and retarded fibrosis. Since then, other possible therapeutic approaches have been explored to reduce collagen accumulation. Some of these approaches have targeted the regulation of tissue inhibitors of metalloproteinases (TIMPs), an inhibitor of collagenase [Shapiro et al., 1992; Pardo et al., 1992], or the production and activation of intracellular collagenases such as matrix metalloproteinases (MMPs) [Horwitz et al., 1977].

Recently, cytokines such as TGF-β and IL-1β have been shown to have prominent effects in the regulation of collagen synthesis. Of these two, TGF-β appears to be the key factor, contributing to the increase in collagen mRNA synthesis [Ritzenthaler et al., 1993; Fine et al., 1990; Diaz et al., 1989]. On the other hand, IL-1β has been shown to both enhance [Elias et al., 1990] and inhibit [Diaz et al., 1993; Armendariz-Borunda et al., 1992] collagen synthesis. Nonetheless, IL-1β seems to have a great potential to facilitate collagen accumulation, for it has been shown to increase LO synthesis [Roy et al., 1996].

TGF-β and IL-1β

During the wound healing process, the blood-born monocytes recruit the macrophages to the wound cleft [Leibovich and Ross, 1975]. Subsequently, macrophages release a battery of growth factors as well as the cytokines, such as TGF-β and IL-1β, at the wound cleft. These cytokines are also synthesized and released by surrounding connective tissue cells [Bienkowski and Gotkin, 1995; Gauldie et al., 1993] and endothelial cells [Phan et al., 1991].

The cellular response during wound healing appears to parallel the fibrotic response. In rats, the intratracheal administration of bleomycin elicits an acute inflammatory response in the lung followed by the development of pulmonary fibrosis. However, the mechanism by which bleomycin causes fibrosis is not understood. It has been proposed to involve the pulmonary macrophage which is considered a central cell in the cytokine network of the lung [Scheule et al., 1992]. In any case, in a bleomycin-induced pulmonary fibrosis, Breen et al. (1992) showed an increase in the TGF-β mRNA levels in rat lung fibroblasts in culture, while Phan et al. (1991) demonstrated a similar increase of TGF-β mRNA levels in rat lung endothelial cells. Further, Scheule et al. (1992) found using an *in vitro* model that bleomycin also increased IL-1β synthesis and secretion by human alveolar macrophages. Overall, these results seem to suggest that the cytokines, such as TGF-β and IL-1β, could be the key factors in the development of fibrosis.

The TGF-β superfamily constitutes local mediating factors which are classified into three subgroups, and are involved in many cellular processes in vertebrates [Brand and Schneider, 1995]. The three subgroups consist of the five isoforms of TGF-βs (TGF-β1 - β5), activins and bone morphogenetic proteins (BMPs) [Massague, 1990]. All the members of the TGF-β superfamily are first synthesized as large latent complexes which are inactive [Derynck et al., 1985; Purchio et al., 1988]. In the latent form, TGF-β is noncovalently bound to the latency associated peptide (LAP), a homodimer formed from the propeptide region of TGF-β [Miyazono et al., 1988]. The LAP is disulfide linked to latent TGF-β binding protein which has features in common with extracellular matrix proteins, and it targets latent TGF-β to the matrix [Derynck et al., 1985; Miyazono et al., 1988; Munger et al., 1997]. Although the physiologic mechanism for activation of TGF-β latent form is not clear, it has been activated through a cleavage at a conserved RXXR motif in the mature C-terminal dimer and the N-terminal propeptide region [Brand and Schneider, 1995].

There are three known TGF-β receptors, types I, II and III. In most mammalian cells, these receptors have approximate molecular weights of 50 kDa, 80 kDa and 280 kDa, respectively [Brand and Schneider, 1995]. In mutant mink lung epithelial cells, it has been shown that types I and II receptors primarily mediate the signaling of TGF-β [Laiho et al., 1990; Brand and Schneider, 1995]. On the other hand, the type III, binds TGF-β with high affinity, but has no

apparent signaling function [Brand and Schneider, 1995]. The type I and II receptors, both have N-terminal cysteine-rich extracellular domains followed by hydrophobic single-pass transmembrane domain and serine-threonine kinase intracellular domains at the C-terminus [Wrana and Pawson, 1997; Padgett et al., 1997]. However, unlike the type II receptor, the type I contains a glycine-serine-rich region immediately preceding the kinase domain. Phosphorylation of this region has been implicated in the signaling activity [Padgett et al., 1997].

One TGF-β signaling model consists of TGF-β activation by release of the mature growth factor from its LAP. The mature TGF-β then binds to the type II receptor which phosphorylates and activates the type I receptor, which subsequently activates an intracellular signaling cascade [Wrana and Pawson, 1997; Padgett et al., 1997]. Currently, the signaling mechanisms involving other combinations of the receptor types are still being explored. Recently, the discovery of a TGF-β downstream signaling protein, a mammalian Mad (*Mothers against decapentaplegic*) related protein called smad, gave a glimpse of how this cytokine induces its signal [de Caestecker et al., 1997; Heldin et al., 1997]. However, there has been no indication of such protein being involved in the TGF-β signaling mechanism responsible for collagen synthesis.

IL-1 has been known as "endogenous pyrogen" as early as the 1940's for its ability to induce fever [Dinarello, 1988]. The IL-1 gene family has three members, IL-1α [Lomedico et al., 1984], IL-1β [Auron et al., 1984] and IL-1 receptor antagonist (IL-1Ra) [Seckinger et al., 1987; Hannum et al., 1990].

Although IL-1α and IL-1β in humans have shown only about 26% amino acid sequence homology, both bind to the same receptors with similar affinities [Kuno and Matsushima, 1994]. The two are also similar in their translational and post-translational modifications. Through immunoelectron microscopy, Stevenson et al. (1992) detected that the IL-1α and IL-1β, 31kDa precursor molecules, were translated on cytoskeletal-associated free polyribosomes in human monocytes. Also, both lack leader sequences and undergo myristolylation on lysine residues in their N-terminal propieces [Stevenson et al., 1993].

The precursor molecule of IL-1α is fully active, and has been known to remain intracellularly [Dinarello, 1996]. It has also been found to translocate into the nucleus and bind to DNA [Maier et al., 1994]. Such nuclear targeting was due to the basic cluster, KVLKKRR, at the N-terminus of IL-1α precursor. However, IL-1α precursor can be cleaved to become 17kDa molecule either by the activation of the calcium dependent, membrane associated cysteine-rich proteases called calpains [Kobayashi et al., 1990] or by the extracellular proteases [Kobayashi et al., 1991]. In contrast, IL-1β precursor must be enzymatically cleaved by IL-1β cleaving enzyme (ICE) in the cytosol before it can become active [Black et al., 1988; Walker et al., 1994]. After cleavage, the 17kDa, mature IL-1β is secreted into the extracellular space through a putative membrane [Dinarello, 1996].

While IL-1α and IL-1β are agonists for IL-1 receptors [Alheim et al., 1997], the IL-1Ra is an antagonist [Arend et al., 1990]. Unlike IL-1α and IL-1β, IL-1Ra is

translated in the endoplasmic reticulum with a leader sequence and transported to the Golgi apparatus [Dinarello, 1996]. Subsequently, it is released into the extracellular space upon cleavage of the leader sequence. Using a murine recombinant IL-1Ra in human synovial cells and rabbit articular chondrocytes, Arend et al. (1990) showed that IL-1Ra inhibited IL-1α and IL-1β induced PGE_2 production in a dose dependent manner. Further, the same experiment showed that the collagenase production by the synovial cells was also inhibited.

Although IL-1Ra is an antagonist, its binding affinity to the IL-1 receptors is similar to the two agonists. Using a murine thymoma cell line, Dripps et al. (1991) showed that IL-1Ra bound to the 80 kDa IL-1 receptor with an affinity (KD = 150 pM) similar to that of IL-1α and IL-1β. The amino acid sequence homology of the IL-1Ra to the mature, or activated, IL-1β is about 26% [Dinarello, 1996].

There are two types, type I (80kDa) and II (60-68kDa), of IL-1 receptors that bind to IL-1α and IL-1β [Kuno and Matsushima, 1994]. The two receptors have single-membrane spanning segments and the ligand binding domains which possess three immunoglobulin-like domains [Dinarello, 1996]. These receptors also exist in soluble forms and have been found in inflammatory synovial fluid and in other pathologic body fluids [Arend et al., 1994].

The type I receptor is considered the main receptor involved in IL-1 signal transduction while the type II receptor is known as a "decoy receptor". Binding of IL-1 to the type II receptor does not generate signal [Colotta et al., 1993]. One of the main reasons for the difference in their signaling abilities seems to be in their

cytoplasmic regions. While the cytoplasmic domain of the type I receptor in human contains about 215 amino acids, the type II cytoplasmic domain only consists of 29 amino acids [Kuno and Matsushima, 1994]. Nevertheless, both receptor genes share the same chromosomal locations, 2q12-22 [Kuno and Matsushima, 1994].

The signal transduction by IL-1 receptor appears to require the binding of IL-1 to the type I receptor and subsequent binding of this complex to the IL-1 receptor-accessory protein (IL-1RAcP) [Greenfeder et al., 1995]. The IL-1RAcP is a single-membrane spanning segment as the type I receptor, and the cytoplasmic domains of both IL-1RacP and type I receptor contain the same amino acid domains commonly found in the GTPase superfamily [Hopp, 1995]. Although their signaling mechanism is still being explored, the heterodimer complex, formed by IL-1 receptor and IL-1RAcP, is likely to initiate the IL-1 signaling cascade through the association of its cytoplasmic domain with GTPase activating proteins [Dinarello, 1996]. The IL-1RAcP does not form a complex with either the ligand-bound type II receptor or IL-1Ra bound to the type I receptor [Dinarello, 1996].

In a number of experiments, IL-1 has been shown to effect PGE_2 synthesis and cAMP production. In the human lung fibroblast cell line, TIG-1, the IL-1α or IL-1β increased PGE_2 synthesis and secondary cAMP production [Takii et al., 1992]. In the same experiment, indomethacin, a COX inhibitor, inhibited the IL-1 induced PGE_2 synthesis and adenosine 3',5'-cyclic monophosphate

(cAMP) production. Kol et al. (1997) also showed IL-1β increased arachidonic acid release, prostanoid accumulation and cytosolic phospholipase A_2 (PLA_2) transcripts as well as PLA_2 activities. These effects all contribute to the PGE_2 production. Similarly, Roy et al. (1996) showed IL-1β treated IMR90 cells increased PGE_2 synthesis by more than 5-fold above the control level. Additionally, IL-1β increased COX-1 and COX-2 mRNA expressions by 3-fold and by more than 5-fold, respectively [Roy et al., 1996].

COX and PGE_2

Prostaglandins and related fatty acid derivatives are one of the naturally occurring autocoids that seem to induce a variety of cellular responses. Under physiological conditions, prostaglandin synthesis takes place following the liberation of precursor fatty acid arachidonate from the membrane lipids and other lipid esters by phospholipases [Lands and Samuelsson, 1968]. Subsequently, the arachidonate is transformed into prostaglandin H_2 by the enzyme prostaglandin endoperoxide H synthase (PGHS) and PGE_2 is formed from PGH_2 by prostaglandin E synthase [Samuelsson et al., 1975].

The PGHS is a single protein with two catalytic sites, cyclooxygenase and peroxidase active sites. The cyclooxygenase converts arachidonate into unstable PGG_2 through oxygenation and cyclization of a pentane ring [Samuelsson, 1975]. Then the peroxidase reduces PGG_2 to PGH_2. There are two isoforms of PGHS, PGHS-1 and -2 which are also referred to COX-1 and COX-2, respectively [Smith and Dewitt, 1996]. These two isozymes exist as

homodimers, heme-containing, glycosylated proteins which are membrane bound. However, both are encoded by separate genes located on different chromosomes, Chromosomes 9 and 1 for COX-1 and COX-2, respectively [Crofford, 1997].

The COX-1 lacks a TATA box which is typical of a housekeeping gene. Also, it is constitutively expressed in most cells [Kraemer et al., 1992]. On the other hand, COX-2 is undetectable in most mammalian tissues, but it can be induced during the state of acute inflammation [Seibert et al., 1994; Vane et al., 1994]. The COX-2 promoter contains a TATA box and other regulatory elements which are common in highly regulated genes [Appleby et al., 1994]. These elements are known to respond during inflammation to nuclear factor-κB, cAMP response element binding protein and the CAAT enhancer binding proteins [Appleby et al., 1994].

Jackson et al. (1993) demonstrated that IL-1β alone increased PGE_2 synthesis in IMR90 by 8-fold, while IL-1β and TGF-β together increased PGE_2 synthesis by 25-fold. In addition, IL-1β and TGF-β increased PLA_2 and COX-1 mRNA, respectively, while IL-1β rapidly induced COX-2 mRNA in fibroblasts [Jackson et al., 1993; DeWitt and Meade, 1993]. In addition, Roy et al. (1996) showed that the half-life of COX-2 mRNA ($t_{1/2}$ = 30 min) is shorter than COX-1 mRNA ($t_{1/2}$ = 4 h). Also, PGE_2 induced COX-1 synthesis [Roy et al., 1996]. These results suggested that COX-2 may provide the initial catalytic activity required for the increased synthesis of PGE_2 at the beginning of an inflammatory

response. Subsequently, PGE_2 could increase the synthesis of COX-1 which may participate in maintaining the elevated level of PGE_2 [Roy et al., 1996]. Overall, the results from Jackson et al. (1993) and Roy et al. (1996) suggest that perhaps the increase in PGE_2 synthesis in response to cytokines, IL-1β and TGF-β, could very well be due to the coordinated regulation of the COX-1 and COX-2.

Although the functional importance of COX isozymes are still being elucidated, their importance has been noted in idiopathic pulmonary fibrosis. According to Wilborn et al. (1995), lung fibroblasts isolated from the patients with idiopathic pulmonary fibrosis showed decreased level of COX-2 expression compared to the normal lung fibroblasts. Consequently, the diseased fibroblasts' capacity to synthesize PGE_2 was diminished.

PGE_2 Receptors

PGE_2 exerts its diversified biological action on cells in an autocrine fashion utilizing four PGE_2 receptor (EP) subtypes, EP1 [Funk et al., 1993], EP2 [Regan et al., 1994], EP3 [Regan et al., 1994], and EP4 [An et al., 1993; Bastien et al., 1994]. These receptors are G-protein coupled, seven transmembrane receptors [Coleman et al., 1994; Ichikawa et al., 1996]. EP1 receptors have been found to be coupled to increases in intracellular Ca^{2+} in Chinese hamster ovary (CHO) cells [Katoh et al., 1995] and COS cells [Funk et al., 1993]. On the other hand, both EP2 and EP4 receptors have been known to stimulate adenylate cyclase, thus increasing cAMP [Coleman et al., 1994; Regan et al., 1994; Bastien et al., 1994; Mori et al., 1996; Nishigaki et al., 1996].

However, there are some differences between the EP2 and EP4 receptors, both structurally and functionally. For example, in the cloned mouse EP4 receptor, there are about 3.5 fold more serine and threonine residues in the C-terminal than the cloned mouse EP2 receptor [Nishigaki et al., 1996]. Also, EP4 receptor contains a potential PKA phosphorylation sites whereas none was found on EP2 receptor [Nishigaki et al., 1996]. Such phosphorylation sites may be critical in receptor desensitization [Liggett et al., 1993] and subsequently its signal transduction.

Unlike EP1, EP2 and EP4 receptors, EP3 receptors exist in a number of isoforms. Thus far, six isoforms of EP3 (EP3A, EP3D, EP3E, EP3F, EP3e, EP3f) have been found in human [Pierce et al., 1995]. All cDNAs from human EP3 isoforms showed identical amino acid sequences from their N-terminal to the 11 residues after the putative seventh transmembrane domain [Pierce et al., 1995]. The most obvious difference between the isoforms was the length of the C-terminal, resulting from alternative mRNA splicing [Woodward et al., 1997]. This variance in length may cause the receptor isoforms to function differently. For example, EP3E isoform has the shortest C-terminal region, consisting of only six amino acids following the splice site, and there are no serine or threonine residues. In contrast, EP3D isoform has the longest C-terminal region with 29 amino acids following the splice site and has seven serine and threonine residues [Pierce et al., 1995]. Lefkowitz (1993) noted that the phosphorylation of serine and threonine residues appeared to be involved in G-protein coupled

receptor desensitization. Further, An et al. (1994) reported that PGE_2 induced EP3E isoform desensitization was rapid and transient while EP3D isoform demonstrated slow and persistent desensitization.

The functions of EP3 isoforms are not only diversified, but also contradicting, sometimes. All human EP3 isoforms expressed in baby hamster kidney (BHK) cells inhibited adenylate cyclase activity upon stimulation with PGE_2 [Schmid et al., 1995]. Of all the isoforms, EP3A isoform was most efficient in inhibiting adenylate cyclase. However, overexpression of EP3A isoform also increased cAMP levels in response to PGE_2. Kotani et al. (1995) also showed that some human EP3 isoforms in COS-7 and CHO cells raised cAMP levels when incubated with EP3 agonist, M&B-28767. In addition, the human EP3A, B and C isoforms expressed in BHK cells increased intracellular Ca^{2+} in response to PGE_2 [Schmid et al., 1995]. Moreover, both human [An et al., 1994] and mouse [Irie et al., 1994] EP3 isoforms in CHO cells, increased intracellular Ca^{2+} in varying degrees and decreased cAMP levels.

Cyclic AMP

Cyclic AMP is an intracellular mediator that is synthesized from ATP by the enzyme adenylate cyclase. To date, 11 isoforms of adenylate cyclase have been found in mammalian cells [Houslay and Milligan, 1997]. This enzyme is activated by the Gsα subunit of trimeric G-protein.

The cyclic AMP plays a vital role in signal transduction of many G protein coupled, seven transmembrane receptors. Increases in intracellular cAMP levels

results in various cellular processes such as protein metabolism, secretory, pro-inflammatory cytokine production, apoptosis and growth control [Houslay and Milligan, 1997]. For example, the increase in cAMP level by parathyroid hormone in rat osteoblastic osteosarcoma cells, decreases collagen mRNA synthesis and mitogenesis of the cells [Pun, 1989]. Such losses may contribute to the loss of bone in hyperparathyroidism.

The effects derived from increased levels of cAMP production usually involve the cAMP-dependent protein kinase, known as protein kinase A (PKA) [Krebs, 1989]. The PKA consists of tetramers containing two regulatory (R) subunits and two catalytic (C) subunits [Nesterova et al., 1996]. Upon binding of cAMP, the two C subunits dissociate from the R subunits and become active, phosphorylating target proteins. There are a number of mammalian isoforms for both subunits: RIα, RIβ, RIIα, RIIβ, Cα, Cβ, Cγ [Nesterova et al., 1996; Dell'Acqua and Scott, 1997]. Each isoform of R subunit can associate with any of the C subunits while different R subunits can heterodimerize together [Doskeland et al., 1993; Nesterova et al., 1996; Dell'Acqua and Scott, 1997]. While C subunit isoforms show no distinguishable difference in substrate specificity and interaction with either R subunits, the dimeric R subunits show distinct cAMP binding affinities and different localization in the cells [Taylor et al., 1990].

Additionally, the effects of cAMP are further modulated through the control of cAMP phosphodiesterase (PDE) activity [Houslay and Milligan, 1997]. The

cAMP PDE hydrolyzes cAMP to 5'AMP, thus inactivating cAMP activities. To date, over 30 isoforms of PDE have been reported for mammalian cells [Houslay and Milligan, 1997].

A number of hormones mediate their signals through cAMP. How these hormones activate their respective signaling pathways by inducing the correct pool of enzymes, which includes adenylate cyclase, PKA and/or cAMP PDE, is still not clear. Recently, a "targeting hypothesis" has been proposed in response to this phenomenon [Dell'Acqua and Scott, 1997]. This hypothesis proposes that the activation of a specific enzyme is determined by its association with targeting subunits, now known as anchoring protein [Johnson et al., 1997; Visconti et al., 1997; Feliciello et al., 1997; Pawson and Scott, 1997; Dell'Acqua and Scott, 1997].

Interestingly, one of the signaling mediators reported to influence collagen synthesis is cAMP. Saltzman et al. (1982) and Baum et al. (1978) showed increased levels of cAMP suppressed collagen synthesis. In contrast, Fine et al (1992) reported that the inhibitory effect of PGE_2 in collagen synthesis was not mediated through cAMP.

Overall, the pathologic hallmark of tissue scarring and fibrosis is collagen accumulation. TGF-β and IL-1β increase collagen and LO mRNAs, respectively. In combination or alone, these cytokines also increase COX-1 and COX-2 expressions, and PGE_2 synthesis. PGE_2 has shown to abrogate both collagen and LO mRNA expressions, even in the presence of the two cytokines. The

effect of PGE_2 is clearly inhibitory in both collagen synthesis and deposition. PGE_2 in essence may play the central role in this process. However, the mechanism involved in such inhibition is not yet clear. The interaction between PGE_2 and its receptor subtypes activate signal transduction pathways that are independent and sometimes contradictory in nature. Nonetheless, the elucidation of this complicated signaling mechanism could provide valuable information leading to limitation or inhibition of tissue scarring and/or fibrosis.

MATERIALS AND METHODS

Materials

PGE_2 and forskolin were purchased from Sigma (St Louis, MO); IL-1β was purchased from Genzyme (Cambridge, MA); sulprostone and TGF-β were purchased from R & D Systems Inc. (Minneapolis, MN); and 11-deoxy PGE_1 was purchased from Cayman Chemical Co (Ann Arbor, MI). EP1 cDNA was a gift of Dr. C.D. Funk (University of Pennsylvania); EP2, and EP3 were gifts of Dr. J. W. Regan (University of Arizona); EP4 cDNA, and COLα1 cDNA were the gifts of Dr. E. J. Goetzl (University of California Medical Center at San Francisco) and Dr. RH Goldstein of Boston University School of Medicine, respectively. Human COX-1 cDNA was a gift from Dr. T. Hla, Holland Laboratory, American Red Cross; and human LO cDNA was a gift from Dr. H. Kagan of Boston University School of Medicine.

Methods

Cell culture IMR90 cells were obtained from the Coriell Institute for Medical Research (Camden, NJ). The cells were grown to confluence in 100x20 mm sterile Falcon tissue culture plates for Northern blot experiments, and in a 12-well plates for cAMP assay. These cells were cultured at 37°C in Minimum Essential Medium Eagle (MEM; Mediatech, Inc., Herndon, VA) containing 10% fetal bovine serum (FBS) supplemented with 50units/ml penicillin and 50μg/ml streptomycin. Cells were used at population doubling level of 18 to 30. Before initiation of experiments, the cells were maintained in MEM containing 0.5% FBS for 24 h,

and incubated with or without (control) test agents for 18 h for Northern blot experiments and 30 min for cAMP assays.

Rat-1 cells were obtained from Dr. Robert Weinberg (Whitehead institute, MIT). Cells were grown to confluence in 100x20 mm sterile Falcon tissue culture plates at 37°C in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies Grand Island, NY) containing 5% FBS supplemented with 50units/ml penicillin and 50µg/ml streptomycin. The cells were incubated with or without (control) test agents for 18 h for Northern blot experiments and 30 min for cAMP assays.

cDNA Probe preparation

EP1 cDNA isolation from pcDNA-1 For transformation of EP1 cDNA in pcDNA-1 (Invitrogen, Carlsbad, CA), 100µl of competent cells, MC 1061/p3 (Invitrogen), was thawed on ice. Then, 5µl of 0.5M β-mercaptoethanol was added and cells were mixed gently. Subsequently, it was further mixed with 0.5µl of the EP1 cDNA in pcDNA-1. The mixture was incubated on ice for 30 min, and at 42°C for 30 s, then back on ice for 2 min. 900µl of SOC media (Invitrogen) was added to the mixture and it was incubated at the 37°C for 1 h. The mixture was centrifuged for 1 min at 12800x g, and all but 50µl of the supernatant was discarded. The pellet was resuspended in remaining 50µl of supernatant. The transformed cells were plated on 95x15 mm plastic petri dish from Fisher Scientific Co. containing Luria-Bertani (LB) agar supplemented with 10mg/ml tetracyclin (TET) and 5 mg/ml ampicillin (AMP), and were stored overnight at 37°C. A colony was then selected and was grown overnight at 37°C in LB with

TET and AMP. The plasmid was prepared by a miniprep procedure. Briefly, 1.2ml of the overnight prep was centrifuged at 12800x g for 2 min. Then the pellet was resuspended using 300µl of TENS (10mM Tris pH 8.0, 1mM EDTA, 0.1N NaOH and 0.4% SDS) and stored in ice for 5 min. Then, 150µl of 3M NaOAc (pH 5.2) was added and the mixture was stored in ice for 8 min prior to centrifuging for 8 min at 12800x g. The supernatant (approximately 450µl) was transferred to a clean tube and precipitated with 2x volume (approximately 900µl) of 100% EtOH. Then, the mixture was centrifuged at 12800x g for 10 min and the pellet was washed two times with 70% EtOH. The pellet was air dried and resuspended in 40µl of TE (10mM Tris Cl pH 8.0, and 1mM EDTA pH 8.0) containing 10µg/ml RNAse. Purified EP1 cDNA in pcDNA-1 was then digested with EcoRI from Stratagene (La Jolla, CA) and electrophoresed on 1x Tris-acetate (TAE) DNA gel (1.2% agarose) along with Lambda DNA-Bst E II digest from New England Biolabs, Inc (Beverly, MA) as a molecular weight standard (1x TAE consists of 0.04M Tris-acetate and 0.001M EDTA). The 1.4-kb band representing EP1 cDNA in the agarose gel was excised and EP1 cDNA was extracted from the gel using the Compass Kit from American Bioanalytical (Natick, MA).

EP2, EP3, EP4, COL, LO and COX-1 cDNA isolation from their vectors

Samples of each cDNA already transformed in JM109 bacterial cells and stored in 50% glycerol at -80°C were amplified by growing them overnight at 37°C in LB supplemented with AMP (50µg/ml). Then, the plasmids were purified using

miniprep procedure as described for EP1 cDNA isolation. The cDNAs were excised from their vectors using specific restriction endonucleases (EP2 – Sac I and Hind III; EP3 – Hind III and Xba I; EP4 – Bam H I and Xba I; COL – Pst I; LO – Eco RI; and COX-1 – Hind III and Xba I). All the restriction endonucleases were obtained from New England Biolabs, Inc.. The digested plasmids were electrophoresed on 1x TAE gel (1.2% agarose) along with Lambda DNA-Bst E II digest as a molecular weight standard. The bands representing respective cDNAs in the agarose gel were excised and extracted from the gel using Compass Kit.

Quantitative analysis 4μl of each purified cDNA was electrophoresed on 1x TAE, DNA gel (1.2% agarose) alongside known concentrations of bradykinin (BK) receptor cDNA to determine the concentration of the newly purified cDNAs. 40ng, 120ng, 200ng, 400ng and 800ng of BK receptor cDNA was run. The intensity of each digested cDNA insert observed through ethidium bromide staining was then compared to the BK receptor cDNA concentrations. From this, the respective stock concentrations for each cDNA from the miniprep were approximated. 30ng of cDNA was used for all probe preparations.

Northern blot analysis Total RNA was extracted from cells by the guanidinium thiocyanate-phenol-chloroform procedure according to the methods described previously [Taylor et al., 1992]. To maximize the RNA concentration, two plates (100x20 mm) of cells were used for each type of incubation for IMR-90 and Rat-1 cells. Each plate of cells was treated with 2ml of Solution D (2M guanidinium-

thiocyanate, 0.04M Na Citrate pH7.0, 207μM β-mercapto ethanol and 0.6% Sarcosyl). Cells were scraped into a sterile Fisher test tube. Subsequently, 240μl of 2M NaOAc, 2ml of phenol (pH 4.5) and 960μl of chloroform were added, vortexing after each addition. The mixture was incubated in ice for 20 min and centrifuged at 9500 rpm (10000x g) for 20 min at 4°C using SS-34 Sorvall centrifuge. Then the top layers (approximately 2ml) were carefully transferred to new tubes using 1ml pipette and the duplicates were pooled in one tube. An equal volume of isopropanol was added to each tube and the mixture was stored at -20°C overnight. On the following day, the mixture was centrifuged at 9750 rpm (10780x g) for 20 min at 4°C. The supernatant was carefully removed with glass pipette to prevent any disturbance of the pellet. The pellet was resuspended with 300μl of Solution D with β-mercaptoethanol and transferred to 1.5ml microfuge tubes. 300μl of isopropanol was added and the tubes vortexed. The mixture was left at -20°C overnight. On the following day, the mixture was centrifuged at 12000 rpm (13500x g) for 25 min at 4°C. The supernatant was discarded and the pellet was washed with 1ml of cold 75% EtOH. The mixture was centrifuged for 5 min at 12000 rpm at 4°C and washed again with another 1ml of 75% EtOH. The mixture was centrifuged for 10 min at 12000 rpm (13500x g) at 4°C. EtOH .was discarded and the pellet was air dried for approximately 2 min. About 35-40μl of DEPC treated H_2O was added to each tube and the pellets were left to dissolve on ice for about 2 h before storing in -80°C. For quantitation, 4μl of RNA was added to 996μl of H_2O and ultraviolet absorbance

was read at 260nm. For RNA gel, 10μg of total RNA was fractionated by electrophoresis on a 1% agarose and 8% formaldehyde denaturing gel. The fractionated RNA was transferred to nylon membranes from NEN Life Science Products (Boston, MA) and fixed by ultraviolet crosslinking. Approximately 30ng of cDNAs for EP receptors (EP1, EP2, EP3 and EP4), COLα1, LO, and COX-1 were labeled with ^{32}P using Ready-to-Go DNA Labeling beads (-dCTP) from Pharmacia Biotech (Piscataway, NJ). The RNA blots were probed with the ^{32}P-labeled cDNA probes at 65°C in a Rapid-hybridization buffer from Amersham Life Science (Amersham, UK.) and washed with 2x SSC buffer (0.3M NaCl, 0.03M Na Citrate, 1% SDS) at 28°C for low stringency then with 0.2x SSC buffer (0.03M NaCl, 0.003M Na Citrate, 0.1% SDS) at 50°C for high stringency. Kodak Scientific Imaging Film from Eastman Kodak Company (Rochester NY) was exposed to the ^{32}P-labeled nylon membrane in –80°C for autoradiography. Radioactivity associated with each band was quantitated with an Instantimager, an electronic autoradiography program (Packard, Meriden, CT). The relative intensity of hybridization with COX-1 probe in Figure 9 was measured using Sigma Scan/Image Program (Jandel Scientific, San Rafael, CA). The cpm values were corrected to eliminate loading errors as follows. The ethidium bromide stained 18s rRNAs were scanned through a video snapshot program (Snappy, Play Inc., Rancho Cordova, CA). The intensities of the ethidium bromide stained 18s rRNAs were measured using a Sigma Scan/Image Program (Jandel Scientific, San Rafael, CA). The cpm values were then divided by the 18s rRNA

values. To normalize the results, values were expressed as either % of control or fold increase.

cAMP assay The cells were labeled with ^{3}H-adenine for 24 h. After labeling, cells were washed with DMEM buffered with 20mM HEPES at pH 7.5. The cells were then incubated in 20mM HEPES buffered DMEM containing 100mM 3-isobutyl-1-methylxanthine (IBMX) and with or without test agents for 30 min. Reactions were stopped by aspirating the medium and adding 1ml ice cold 5% TCA containing 1mM ATP and 1mM cAMP. After 30 min incubation on ice, the TCA extract was collected and applied to Dowex columns containing analytical grade resins, AG 1-x8 Resin (100-200 mesh formate form) from Bio-Rad Laboratories (Hercules, CA). The columns were washed with 3ml of water. A total volume of 4ml was collected in scintillation vials which represented the ^{3}H-ATP and ^{3}H-ADP fractions. Cyclic AMP was then eluted from the Dowex columns directly onto Alumina columns (Sigma Chemical Co., St. Louis, MO) with 10ml water. cAMP was eluted from the Alumina with 6ml 0.1M imidazole, pH 7.5. The radioactivity of the cAMP fraction was quantified by liquid scintillation spectrometry (Wallac, Inc., Gaithersburg, MD).

RESULTS

Effect of PGE_2 on COLα1(I) mRNA in IMR-90

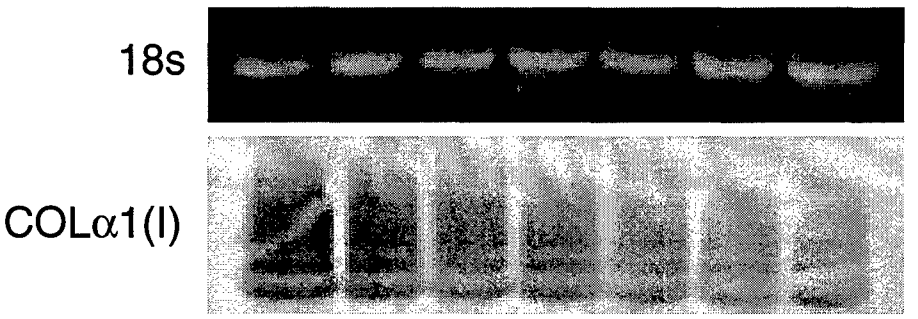
In a concentration dependent manner, PGE_2 reduced COLα1(I) mRNA levels in IMR-90 cells. This is illustrated in Figure 1. The COLα1(I) mRNA level was reduced at 1nM, and further decreased in a stepwise manner up to 1μM. Then, from 1μM to 100μM, the COLα1(I) mRNA level appeared to show no further change. Therefore, PGE_2 induced the maximal inhibition on COLα1(I) at 1μM.

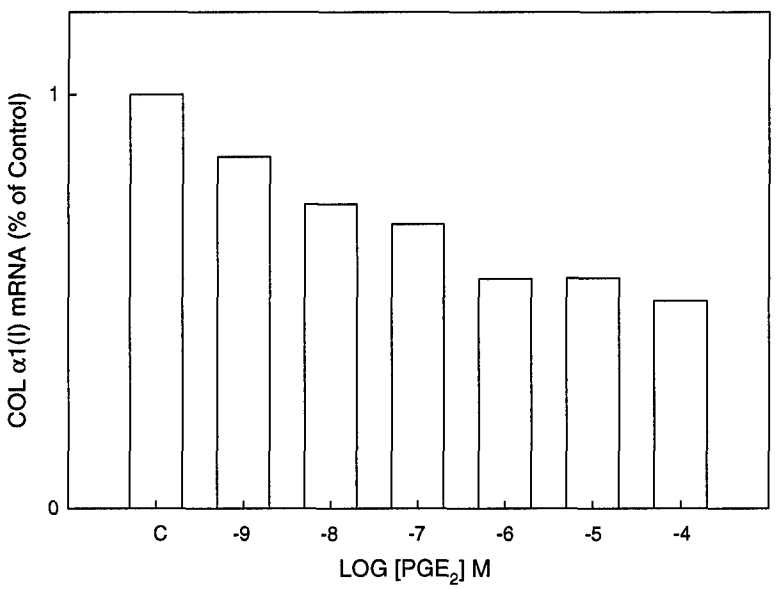
EP Receptor Profile in IMR-90

PGE_2 effects cellular response using the four EP receptors, EP1, EP2, EP3 and EP4 [Coleman et al., 1994; Ichikawa et al., 1996]. Thus, the EP receptor profile in IMR-90 cells was examined (Figure 2). The total RNA was hybridized with EP1, EP2, EP3 and EP4 receptor probes. The expected sizes were approximately 1.6-kb for EP1 [Funk et al., 1993], 3.1-kb for EP2 [Regan et al., 1994], 2.4-kb for EP3 [Regan et al.,1994] and 3.8-kb for EP4 [An et al., 1993]. As shown in Figure 2, the bands for approximate sizes of EP2, EP3 and EP4 were detected. Among these, the EP2 receptor expression appeared the most prominent while the EP3 receptor expression was the least. EP1 receptor expression was not detected. Narumiya (1994) reported similar results in mouse lung in which the expression of EP2 mRNA was predominant compared to the other EP receptors.

Figure 1

Effect of PGE_2 on COLα1(I) mRNA in IMR-90



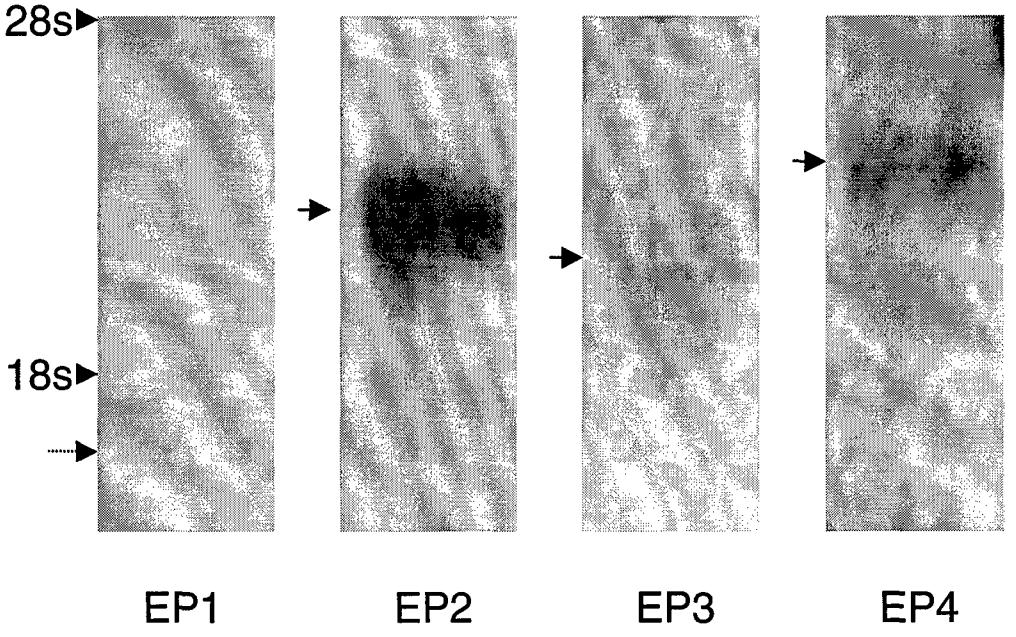


Legend for Figure 1

IMR-90 cells were made quiescent as described in the methods section. The cells were then untreated (control) or treated with the respective concentrations of PGE_2 for 18 h. Total RNA isolated from 2 separately treated 100x20mm culture plates under the same conditions was combined. 10μg of total RNA was fractionated by electrophoresis and transferred to nylon membrane as described in the methods section. 18s rRNA represents the loading. Then, RNA was hybridized with COLα1(I) cDNA. PGE_2 concentrations are expressed as log of molar concentration. C = control.

Figure 2

Northern Blot Analysis of EP receptors
in IMR-90



Legend for Figure 2

Northern blot analysis was performed on 10μg of total RNA isolated from confluent, quiescent, untreated IMR-90 cells. Each lane was probed with specific receptor cDNA. The positions of the 28s and 18s rRNAs are marked with solid arrow heads. The hybridizing transcripts of EP2, EP3 and EP4 are marked with solid-lined arrows (→). The dotted-lined arrow (⇢) represents the expected 1.6-kb hybridizing transcript of EP1 receptor which was reported by Funk et al (1993) and Fedyk and Phipps (1996) in human erythroleukemia cells. Autoradiography was performed at -80°C. The radiogram is a representative of results obtained from three separate experiments.

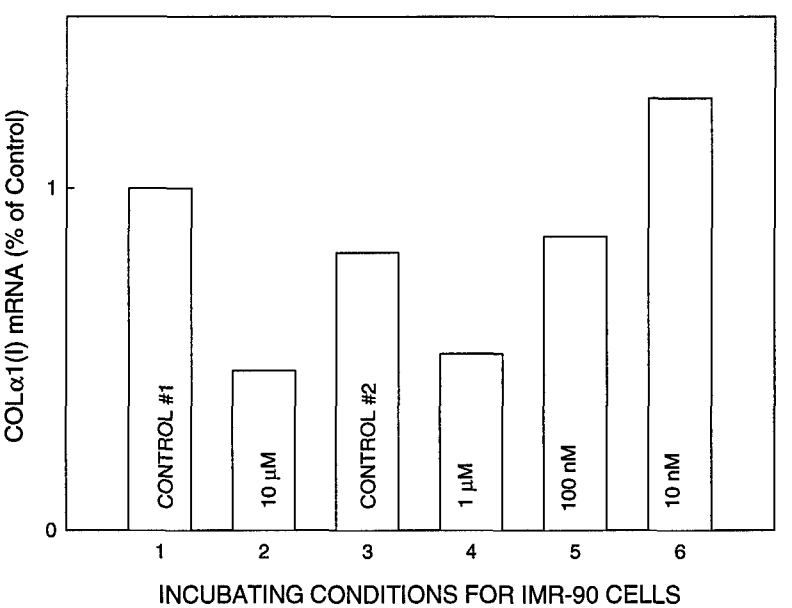
Effect of 11-deoxy PGE_1 and Sulprostone on COLα1(I), LO and COX-1 mRNA in IMR-90

In order to identify the type of EP receptor that was mediating PGE_2 response in IMR-90 cells, the EP2/EP4 agonist, 11-deoxy PGE_1, and EP3/EP1 agonist, sulprostone, were used. The effects of 11-deoxy PGE_1 and sulprostone were examined with respect to COLα1(I) as well as LO and COX-1 mRNA levels.

The Figures 3 - 5 show the effect of varying concentrations (10nM-10μM) of 11-deoxy PGE_1 on COLα1(I) (Figure 3), LO (Figure 4) and COX-1 (Figure 5) gene expression, respectively. 11-deoxy PGE_1 at 10μM decreased the COLα1(I) mRNA level the most, decreasing it by more than 50% from the control. 1μM of 11-deoxy PGE_1 also decreased COLα1(I) mRNA level, but to a lesser degree. However, 100nM of 11-deoxy PGE_1 had no apparent effect while 10nM increased COLα1(I) mRNA level by approximately 30%. In Figure 4, 11-deoxy PGE_1 at 10μM again had the strongest effect in decreasing LO mRNA level. 10μM of 11-deoxy PGE_1 decreased LO mRNA level by 50%. The 11-deoxy PGE_1 concentrations (1μM - 10nM) also decreased LO mRNA levels in a concentration dependent manner and did not show the biphasic effect observed with COLα1(I) shown in Figure 3. From 1μM to 10nM, there was a stepwise decrease in the 11-deoxy PGE_1's inhibitory effect on LO mRNA levels. The effect of 11-deoxy PGE_1 concentrations (1μM - 10nM) on COX-1 mRNA level was opposite from its effect on LO (Figure 5). This figure shows 11-deoxy PGE_1

Figure 3

Effect of 11-deoxy PGE_1 on COLα1(I) mRNA in IMR-90

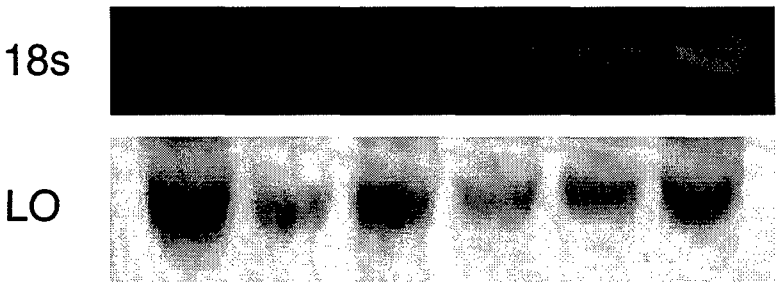


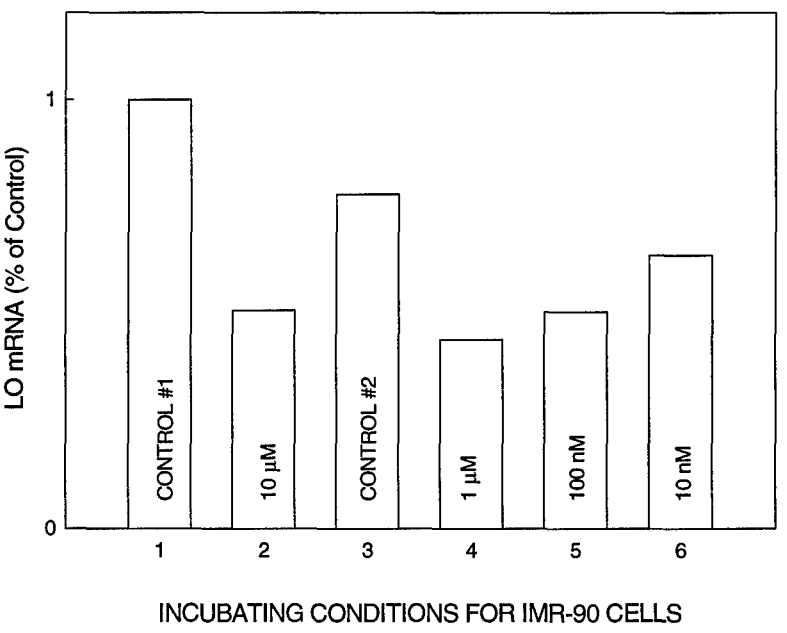
Legend for Figure 3

IMR-90 cells were untreated (control) or treated with 11-deoxy PGE_1 (10μM, 1μM, 100nM and 10nM) for 18 h in MEM containing 0.5% FBS. Total RNA was then isolated as described in the methods section. RNA from two separate incubations of the same condition was then combined. 10μg of total RNA was fractionated by electrophoresis and transferred to nylon membrane as described in the methods section. Then, RNA was hybridized with COLα1(I) cDNA. 18s rRNA represents the loading. Control #1 media is treated with 0.1% ethanol while Control #2 is treated with 0.01% ethanol. The controls were treated with ethanol, for 11-deoxy PGE_1 stock was dissolved in ethanol. There are two controls, for the amount of ethanol in 10μM was different than the amounts for 1μM - 1nM.

Figure 4

Effect of 11-deoxy PGE_1 on LO mRNA in IMR-90



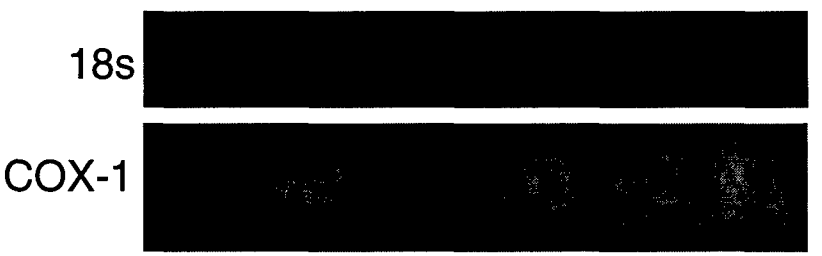


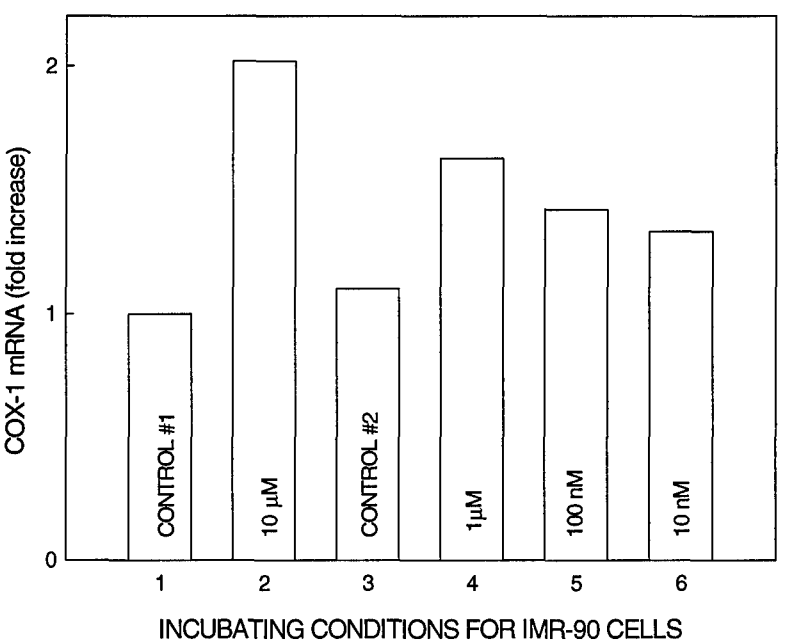
Legend for Figure 4

IMR-90 cells were untreated (control) or treated with 11-deoxy PGE_1 (10μM, 1μM, 100nM and 10nM) for 18 h in MEM containing 0.5% FBS. Total RNA was then isolated as described in methods. RNA from two separate incubations of the same condition was then combined. 10μg of total RNA was fractionated by electrophoresis and transferred to nylon membrane as described in the methods section. Then, RNA was hybridized with LO cDNA. 18s rRNA represents the loading control. Control #1 media is treated with 0.1% ethanol while Control #2 is treated with 0.01% ethanol. The controls were treated with ethanol, for 11-deoxy PGE_1 stock was dissolved in ethanol. There are two controls, for the amount of ethanol in 10μM was different than the amounts for 1μM - 1nM.

Figure 5

Effect of 11-deoxy PGE_1 on COX-1 mRNA in IMR-90





Legend for Figure 5

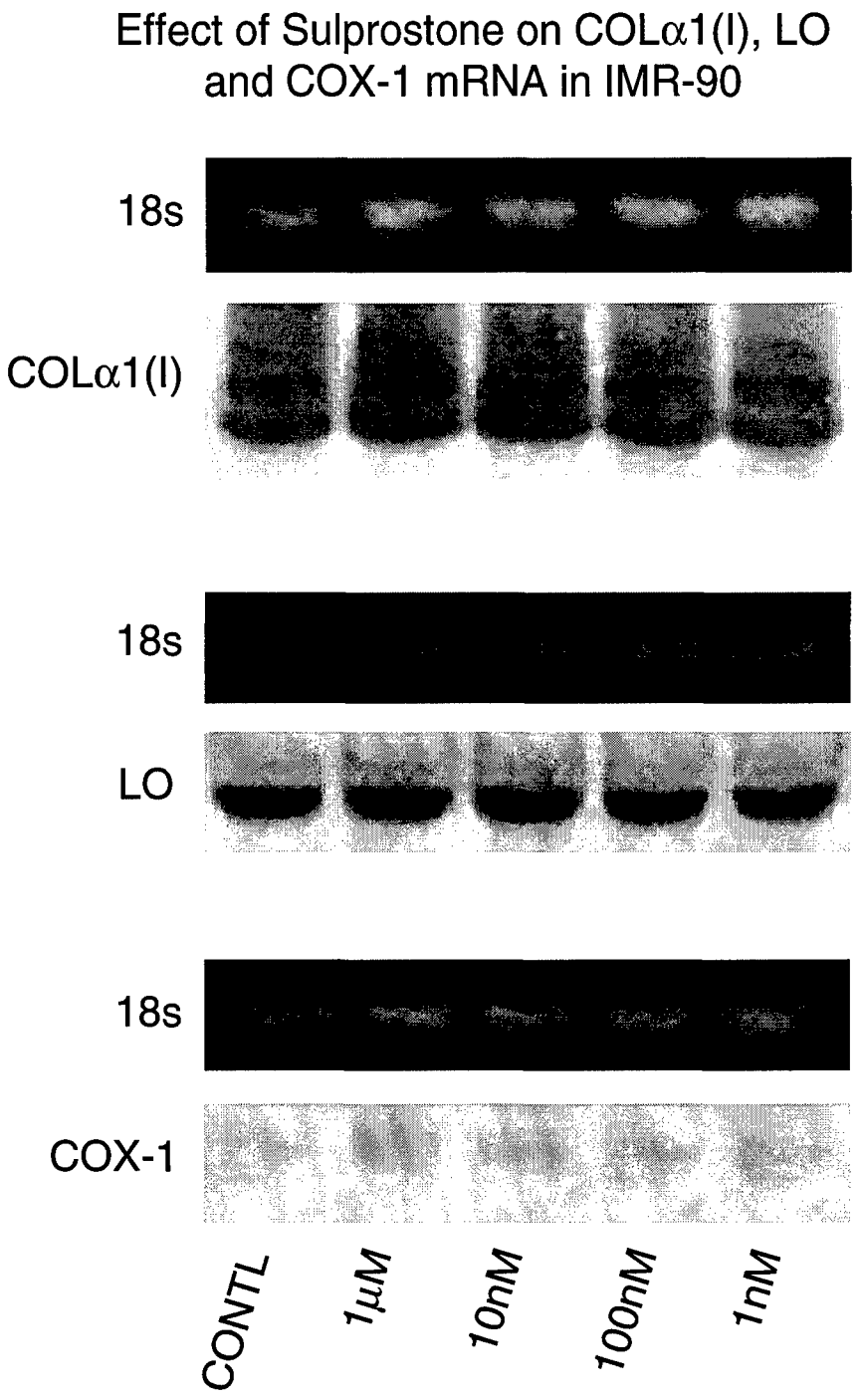
IMR-90 cells were untreated (control) or treated with 11-deoxy PGE_1 (10μM, 1μM, 100nM and 10nM) for 18 h in MEM containing 0.5% FBS. Total RNA was then isolated as described in methods. RNA from two separate incubations of the same condition was then combined 10μg of total RNA was fractionated by electrophoresis and transferred to nylon membrane as described in the methods section. Then, RNA was hybridized with COX-1 cDNA. 18s rRNA represents loading control. Control #1 media is treated with 0.1% ethanol while Control #2 is treated with 0.01% ethanol. The controls were treated with ethanol, for 11-deoxy PGE_1 stock was dissolved in ethanol. There are two controls, for the amount of ethanol in 10μM was different than the amounts for 1μM - 1nM.

increased COX-1 mRNA levels in a concentration dependent manner in which the effect was maximal at 10μM and minimal at 10nM. This figure also did not show the biphasic effect seen in Figure 3. Two different controls were used for Figures 3 - 5. Control #1 was for 10μM 11-deoxy PGE_1 while Control #2 was for 11-deoxy PGE_1 concentrations of 1μM - 10nM. The only difference in the composition of these control vehicles was that there was more ethanol in Control #1 than Control #2. There was some difference between Control #1 and Control #2 in their basal mRNA levels for all three genes. Figure 6 shows the effect of sulprostone on COLα1(I), LO and COX-1 mRNA levels, respectively. In contrast to the effects seen with 11-deoxy PGE_1, sulprostone (1nM-1μM) appeared to have little or no effect on the three genes.

Effect of Forskolin on COLα1(I), LO and COX-1 mRNA in IMR-90

The EP2 and EP4 receptors have been reported to be associated with the cAMP signaling path [Scutt et al., 1995]. Therefore, the effect of forskolin, an adenylyl cyclase activator, was compared to that of PGE_2 on the three genes using IMR-90 cells. These results are shown in Figures 7 - 9. In Figure 7, PGE_2 (1μM) or forskolin (25μM), showed a comparable effect in limiting COLα1(I) mRNA levels. PGE_2 or forskolin decreased COLα1(I) mRNA levels by 50%. Interestingly, when 1μM PGE_2 was co-incubated with 25μM forskolin, the decreasing effect on the COLα1(I) mRNA level was much greater than the effect with either PGE_2 or forskolin, alone. Similarly, as shown in Figure 8, forskolin

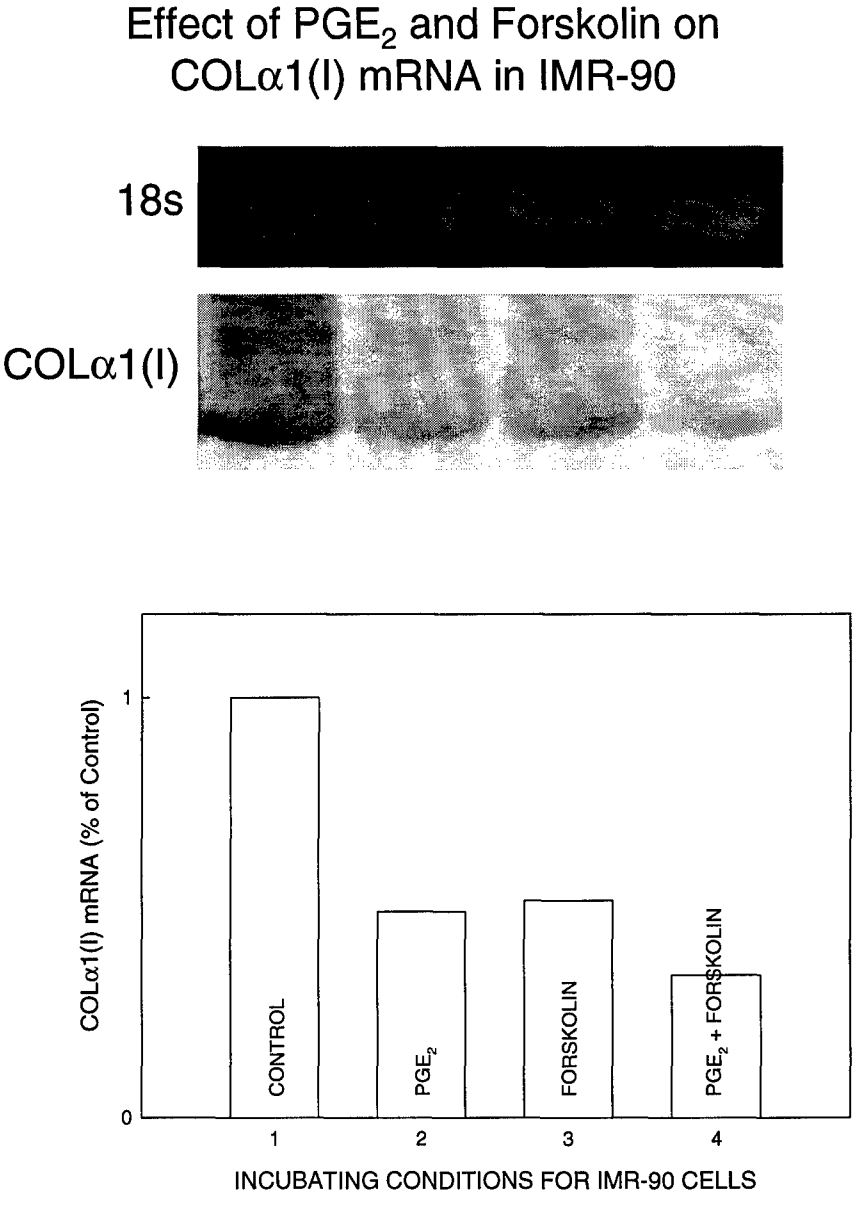
Figure 6


Effect of Sulprostone on COLα1(I), LO and COX-1 mRNA in IMR-90
18s
COLα1(I)
18s
LO
18s
COX-1
CONTL
1μM
10nM
100nM
1nM

Legend for Figure 6

IMR-90 cells were untreated (control) or treated with sulprostone (1μM, 100nM and 10nM) for 18 h in MEM containing 0.5% FBS. Total RNA was then isolated as described in methods. RNA from two separate incubations of the same condition was then combined. 10μg of total RNA was fractionated by electrophoresis and transferred to nylon membrane as described in the methods section. Then, RNA was hybridized with COLα1(I), LO and COX-1 cDNA. 18s rRNA represents the loading control. CONTL = control. 1μM - 1nM = sulprostone concentrations.

Figure 7

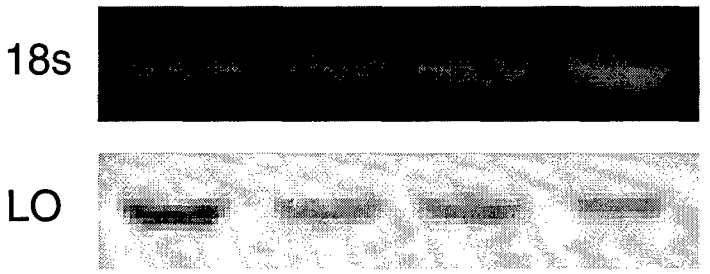

Effect of PGE2 and Forskolin on
COLα1(I) mRNA in IMR-90
18s
COLα1(I)
COLα1(I) mRNA (% of Control)
1
0
CONTROL
PGE2
FORSKOLIN
PGE2 + FORSKOLIN
1
2
3
4
INCUBATING CONDITIONS FOR IMR-90 CELLS

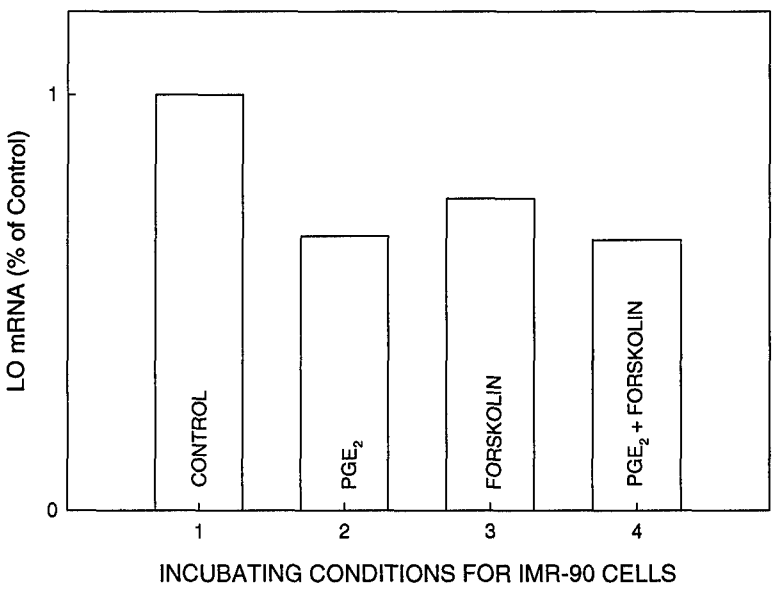
Legend for Figure 7

IMR-90 cells were made quiescent as described in the methods section. The cells were untreated (control) or treated with PGE_2 (1μM) and/or forskolin (25μM) for 18 h. Total RNA was isolated as described in the methods section. 10μg of total RNA was fractionated by electrophoresis and transferred to nylon membrane as described in the methods section. Then, RNA was hybridized with COLα1(I) cDNA. Northern blot analysis is a representative of three separate experiments. 18s rRNA represents the loading control.

Figure 8

Effect of PGE_2 and Forskolin on LO mRNA in IMR-90



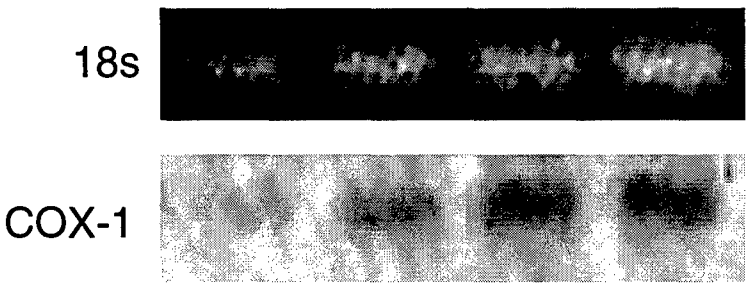


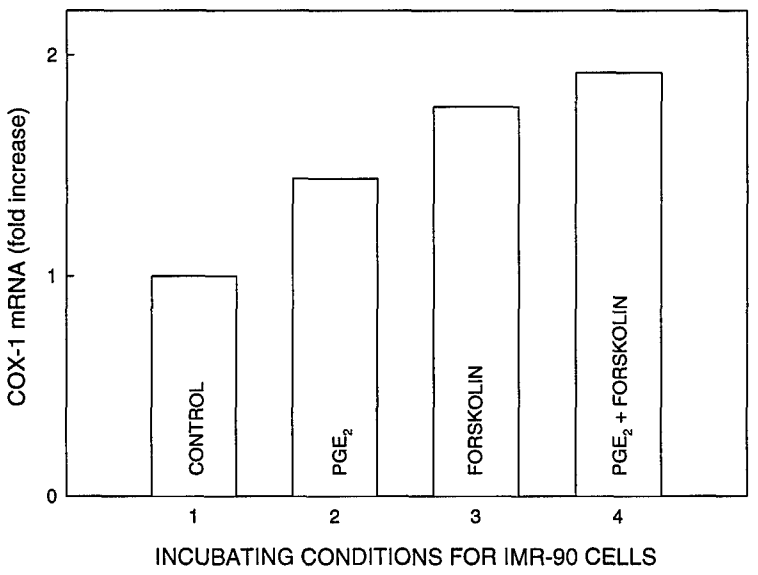
Legend for Figure 8

IMR-90 cells were made quiescent as described in the methods section. The cells were untreated (control) or treated with PGE_2 (1μM) and/or forskolin (25μM) for 18 h. Total RNA was isolated. 10μg of total RNA was fractionated by electrophoresis and transferred to nylon membrane as described in the methods section. Then, RNA was probed with LO cDNA. Northern blot analysis is a representative of three separate experiments. 18s rRNA represents the loading control.

Figure 9

Effect of PGE_2 and Forskolin on COX-1 mRNA in IMR-90





Legend for Figure 9

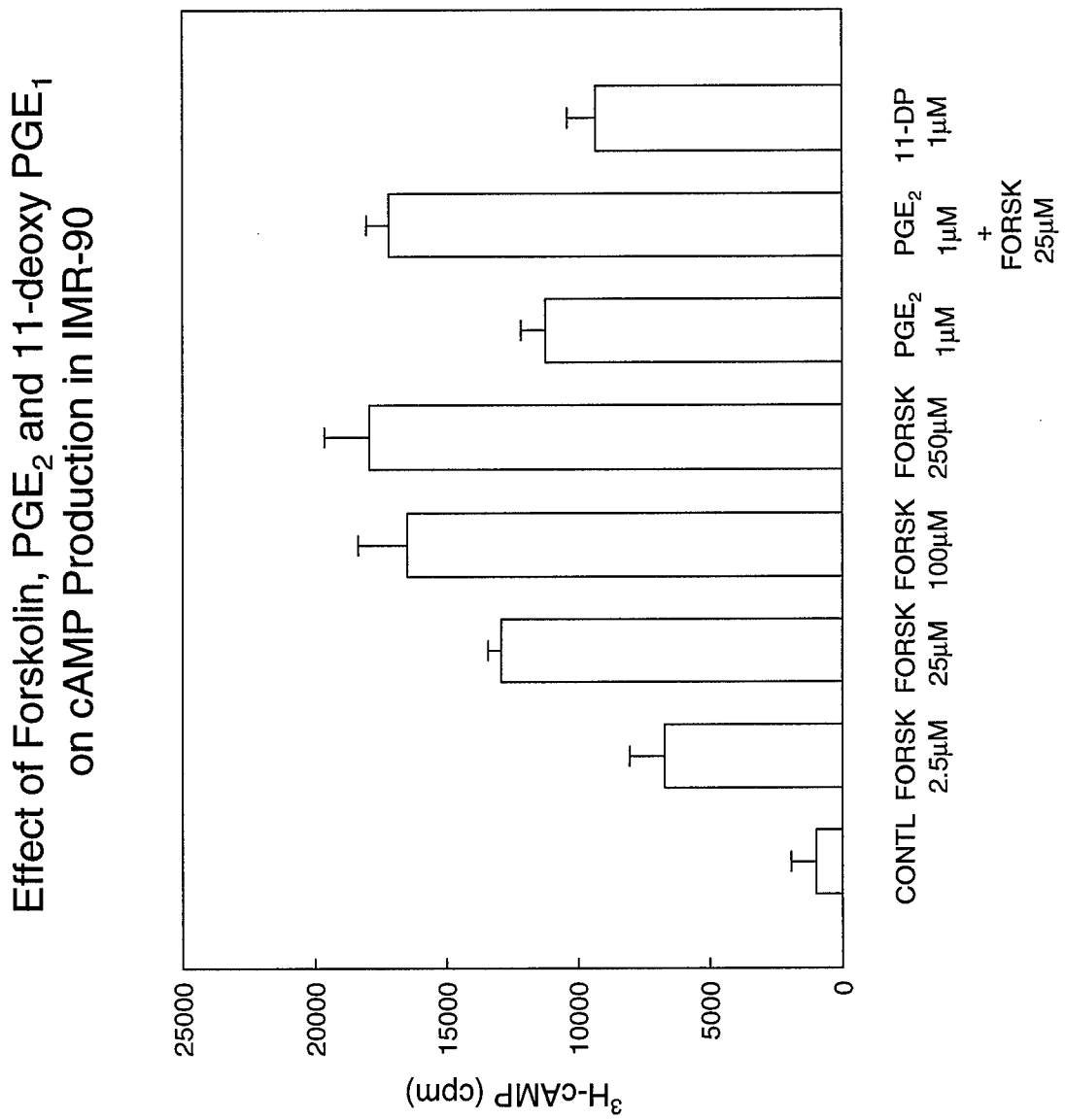
IMR-90 cells were made quiescent as described in the methods section. The cells were untreated (control) or treated with PGE_2 (1μM) and/or forskolin (25μM) for 18 h. Total RNA was isolated. 10μg of total RNA was fractionated by electrophoresis and transferred to nylon membrane as described in the methods section. Then, RNA was probed with COX-1 cDNA. Northern blot analysis is a representative of three separate experiments. 18s rRNA represents the loading control.

(25μM) also decreased LO mRNA levels by approximately 25%, while PGE_2 (1μM) limited LO mRNA slightly more than forskolin. However, no additive effect was observed when the two were combined. In Figure 9, forskolin (25μM) increased COX-1 mRNA levels by more than 50% from the control level. PGE_2 (1μM) increased COX-1 mRNA level by approximately 25%. The co-incubation of PGE_2 and forskolin increased COX-1 mRNA level that was slightly greater than the effect of forskolin. The co-incubation of PGE_2 and forskolin increased COX-1 mRNA levels almost 2-fold above the control.

Cyclic AMP Assay for IMR-90 with Forskolin, PGE_2 and 11-deoxy PGE_1

One explanation for the greater inhibition by PGE_2 in the presence of forskolin was that the dose of either effector alone did not maximize the cAMP levels. This hypothesis was tested with cAMP assay (Figure 10). As shown, forskolin at 2.5μM increased cAMP levels by approximately 5-fold. The increase in cAMP levels paralleled the increase in forskolin concentration. Although forskolin at 250μM increased the cAMP levels the most, it was not much different from the effect at 100μM. In any case, PGE_2 at 1μM also produced sub-optimal increases in cAMP levels. As expected 1μM of PGE_2, co-incubated with 25μM of forskolin, generated the maximal cAMP levels that was comparable to forskolin at 250μM. Finally, since 11-deoxy PGE_1 demonstrated similar effects as PGE_2 on the three genes of interest, 1μM of 11-deoxy PGE_1 was tested to determine its

Figure 10


Effect of Forskolin, PGE2 and 11-deoxy PGE1
on cAMP Production in IMR-90
3H-cAMP (cpm)
25000
20000
15000
10000
5000
0
CONTL
FORSK 2.5μM
FORSK 25μM
FORSK 100μM
FORSK 250μM
PGE2 1μM
PGE2 1μM + FORSK 25μM
11-DP 1μM

Legend for Figure 10

IMR-90 cells were made quiescent and labeled with ^{3}H-adenine as described in the methods section. The cells were treated with IBMX and with or without (control) varying concentrations of forskolin (2.5μM - 250μM), PGE_2 (1μM) with or without forskolin (25μM), or 11-deoxy PGE1 (1μM) for 30 min. ^{3}H-cAMP was eluted and quantitated using liquid scintillation spectrometry. Values for the experiment are the means +/- standard error of three experiments. CONTL = control.

effect on cAMP. 11-deoxy PGE_1 increased cAMP levels, but its effect was slightly less than PGE_2 (1μM) or forskolin (25μM) alone.

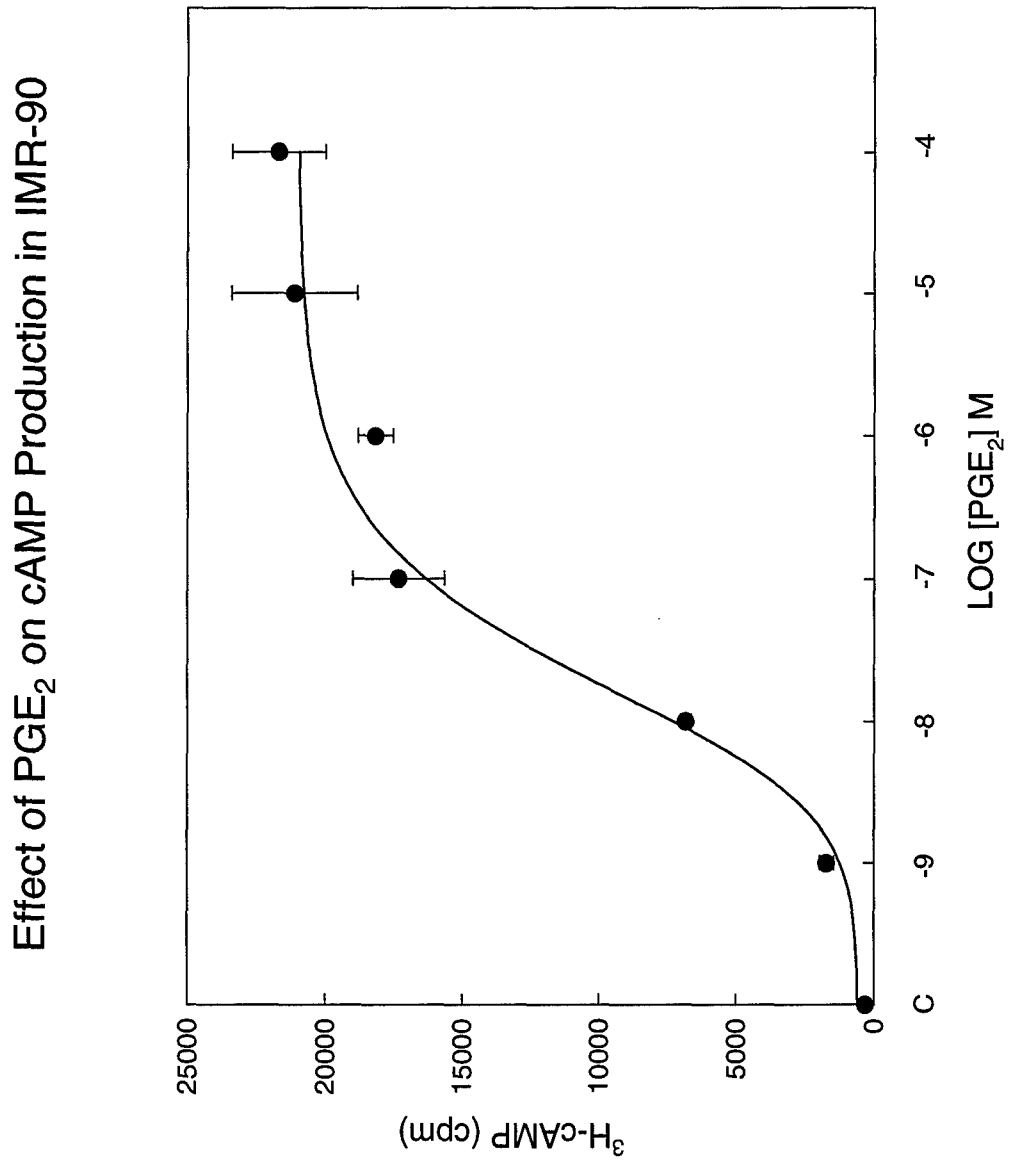
Cyclic AMP Assay for IMR-90 with PGE_2 Concentrations

Since the effect of 1μM of PGE_2 was sub-optimal as shown in Figure 10, the investigator attempted to determine the maximal PGE_2 concentration required for optimal cAMP production in IMR-90 cells (Figure 11). The cAMP levels began to increase even at 1nM of PGE_2. Then, the levels began to plateau at 100nM and were maximized at 1μM. The half-maximal effective dose was $3.32x10^{-8}$nM.

Effect of 11-deoxy PGE_1 on TGF-β and IL-1β stimulated COLα1(I) and LO mRNA levels, respectively, in IMR-90

Since 11-deoxy PGE_1 demonstrated similar effects as PGE_2 on the three genes of interest and on cAMP levels, 11-deoxy PGE_1 was examined to see if it can also limit the cytokine induced increases in COLα1(I) and LO mRNA levels (Figure 12 and 13, respectively). As shown in Figure 12, TGF-β increased the COLα1(I) mRNA levels by about 50%. 11-deoxy PGE_1 decreased the basal and the TGF-β stimulated COLα1(I) mRNA levels by about 50% and more than 60%, respectively. PGE_2 showed almost identical inhibitory effects on the basal and TGF-β stimulated COLα1(I) mRNA levels as 11-deoxy PGE_1. In Figure 13, IL-1β stimulated LO mRNA levels by more than 2-fold from the control. 11-deoxy PGE_1 or PGE_2 decreased the basal LO mRNA levels by about 50%. In addition, 11-deoxy PGE_1 decreased the IL-1β stimulated increase in LO mRNA levels by

Figure 11

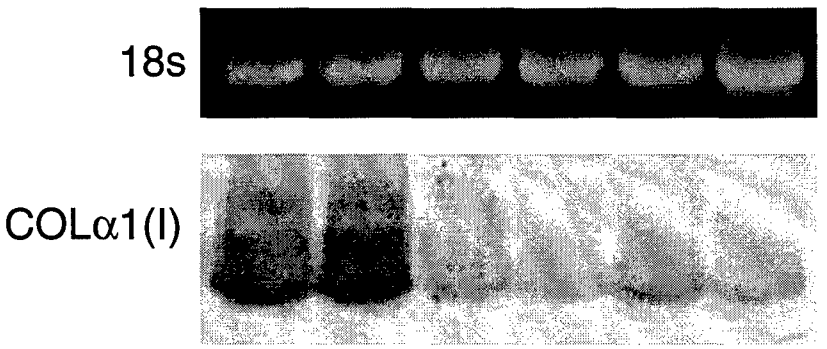

Effect of PGE2 on cAMP Production in IMR-90
3H-cAMP (cpm)
25000
20000
15000
10000
5000
0
C
-9
-8
-7
-6
-5
-4
LOG [PGE2] M

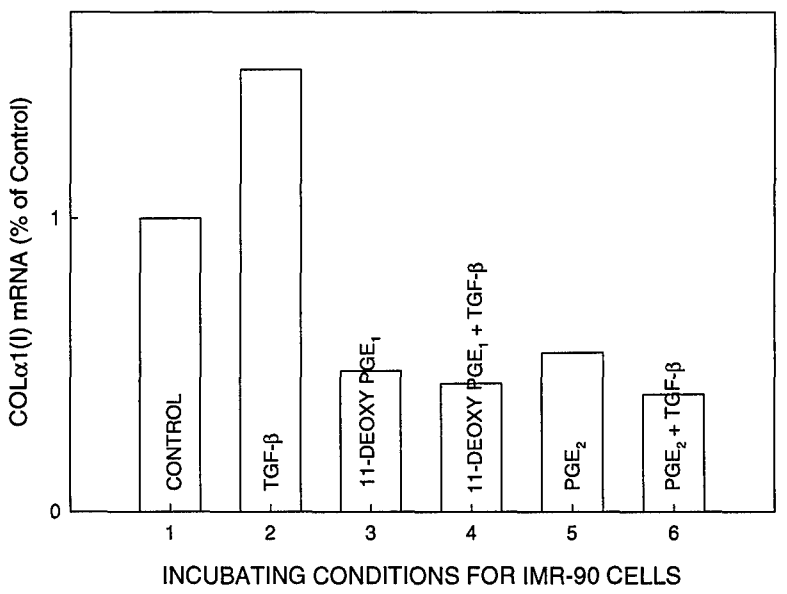
Legend for Figure 11

IMR-90 cells were made quiescent and labeled with ^{3}H-adenine as described in the methods section. The cells were treated with IBMX and with or without (control) varying concentrations of PGE_2 (1nM - 100μM) for 30 min. ^{3}H-cAMP was eluted as described in the methods section and quantitated using liquid scintillation spectrometry. The values for the experiment are the means +/- standard error of three experiments. C = control. 11-DP = 11-deoxy PGE_1.

Figure 12

Effect of TGF-β, 11-deoxy PGE_1 and PGE_2 on COLα1(I) mRNA in IMR-90



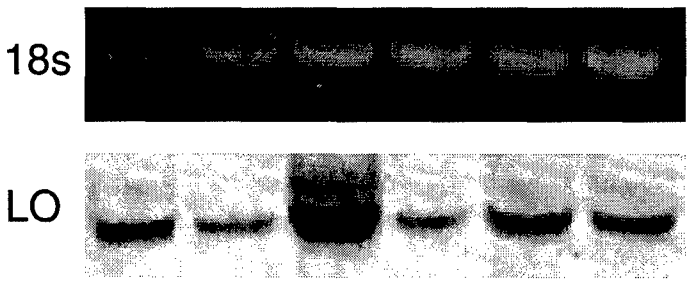


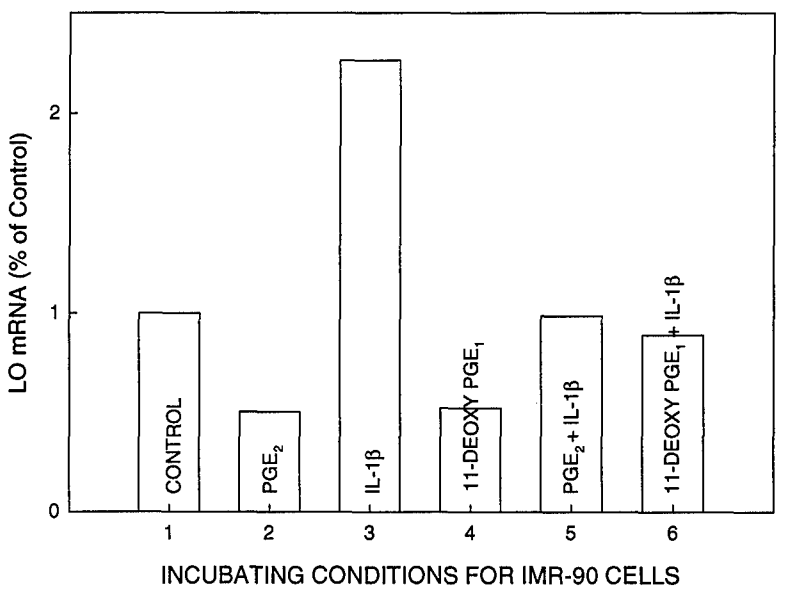
Legend for Figure 12

IMR-90 cells were grown to confluence and made quiescent as described in the methods section. The cells were untreated (control) or treated with PGE_2 (1μM) or 11-deoxy PGE_1 (1μM) with or without TGFβ (100ng/ml) for 18 h. Total RNA isolated from 2 separately treated 100x20mm culture plates was combined. 10μg of total RNA was electrophoresed and transferred to nylon membrane as described in the methods section. Then, the RNA was hybridized with COLα1(I) cDNA. 18s rRNA represents the loading control.

Figure 13

Effect of IL-1β, 11-deoxy PGE_1 and PGE_2 on LO mRNA in IMR-90





Legend for Figure 13

IMR-90 cells were grown to confluence and made quiescent as described in the methods section. The cells were untreated (control) or treated with PGE_2 (1μM) or 11-deoxy PGE_1 (1μM) with or without IL-1β (100pg/ml) for 18 h. Total RNA isolated from 2 separately treated 100x20mm culture plates was combined. 10μg of total RNA was fractionated by electrophoresis and transferred to nylon membrane as described in the methods section. Then, the RNA was hybridized with LO cDNA. 18s rRNA represents loading control.

more than 50%. PGE_2 also decreased the IL-1β stimulated increase in LO mRNA levels to the same extent as 11-deoxy PGE_1.

EP Receptor Profile in Rat-1 Cells

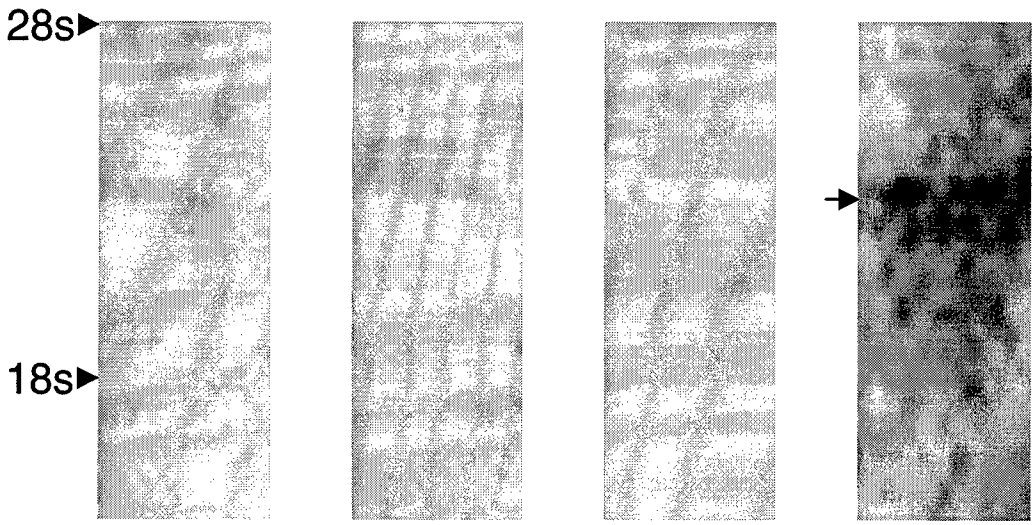
To further test the role of PGE_2 or cAMP in regulating COLα1(I) mRNA levels, another type of cells, Rat-1 cells were tested. These cells actively produce collagen [Granot et al., 1993]. To determine the EP receptor subtype profile in this cell type, total RNA was hybridized with EP1, EP2, EP3 and EP4 receptor probes. Results are shown in Figure 14. Unlike the IMR-90, Rat-1 cells do not express detectable EP1, EP2 or EP3 mRNAs. However, some EP4 receptor mRNA was detected as shown in the figure.

Effect of PGE_2 on COLα1(I) in Rat-1 Cells

Rat-1 cells were incubated with PGE_2 (1μM) or forskolin (25μM) and probed for COLα1(I) mRNA. Results are shown in Figure 15. In contrast to the effects observed in IMR-90 cells, PGE_2 slightly increased COLα1(I) mRNA levels. However, forskolin (25μM) increased COLα1(I) mRNA levels by approximately 80%.

Figure 14

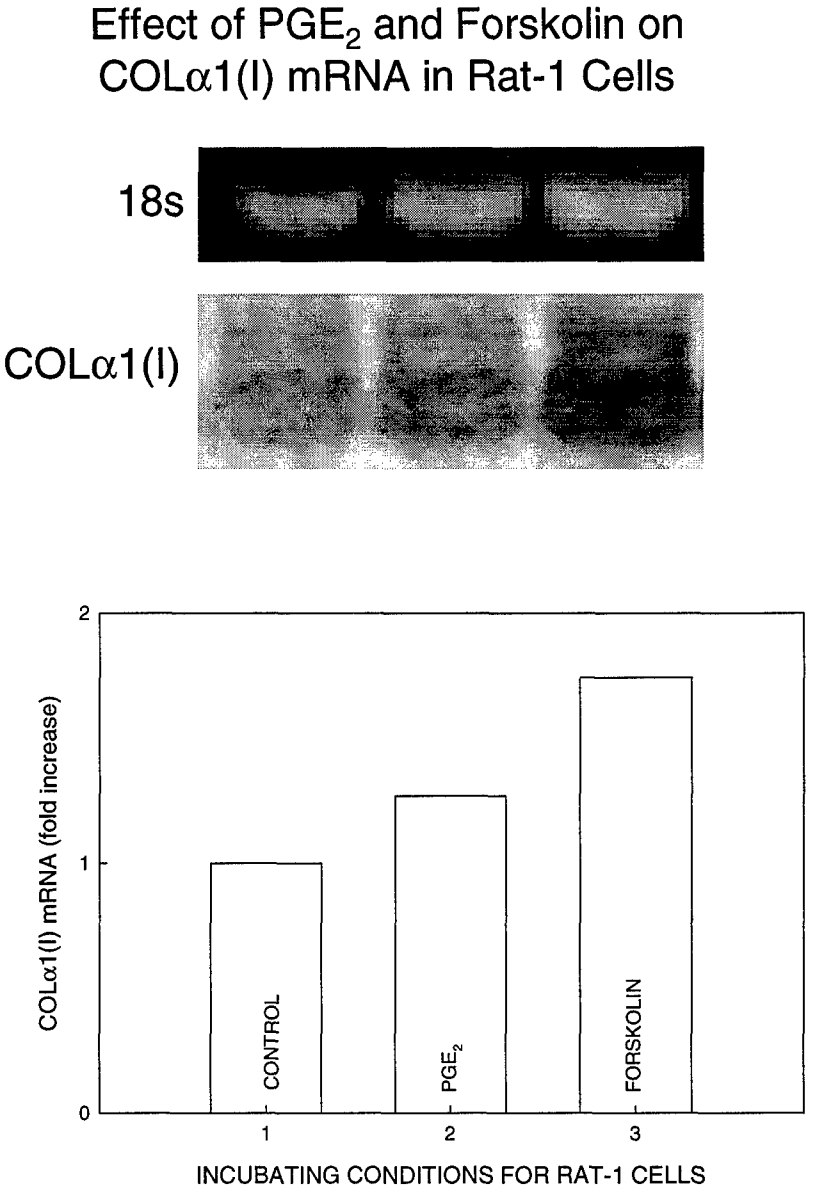
Northern Blot Analysis of EP receptors
in Rat-1 Cells



Legend for Figure 14

Northern blot analysis was performed on 10μg of total RNA isolated from confluent, untreated Rat-1 cells. Each lane was probed with specific type of cDNA. The positions of the 28s and 18s rRNAs are marked with solid arrow heads. The hybridizing transcript of EP4 receptor is marked with solid-lined arrows (→). No band was detected when the blot was probed with for EP1, EP2 or EP3 receptor cDNAs. Autoradiography was performed at -80°C. The radiogram is a representative of results obtained from two separate experiments.

Figure 15


Effect of PGE2 and Forskolin on
COLα1(I) mRNA in Rat-1 Cells
18s
COLα1(I)
2
1
0
COLα1(I) mRNA (fold increase)
CONTROL
PGE2
FORSKOLIN
1
2
3
INCUBATING CONDITIONS FOR RAT-1 CELLS

Legend for Figure 15

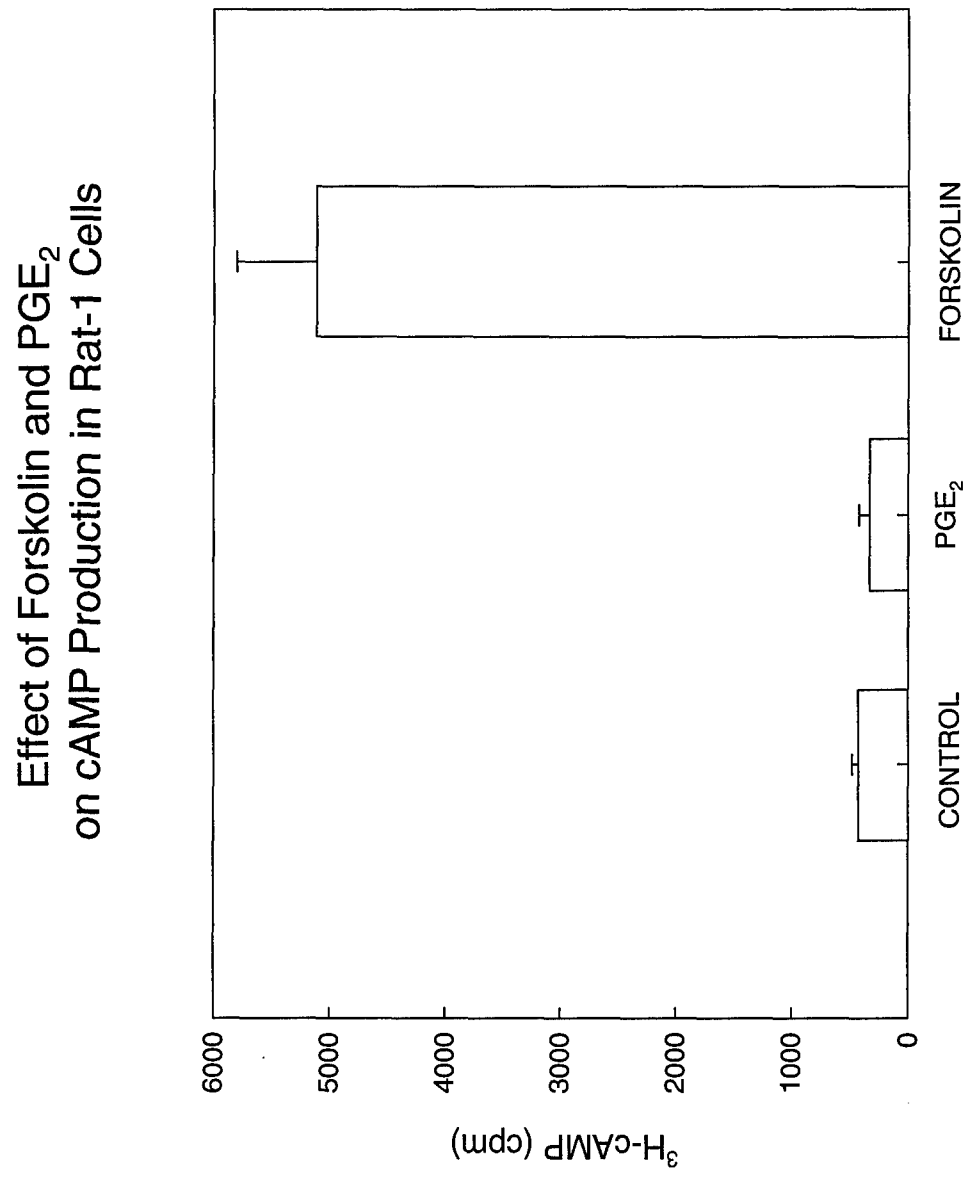
Rat-1 cells grown to confluence were untreated (control) or treated with PGE_2 (1μM) or forskolin (25μM) for 18 h. Total RNA isolated from 2 separately treated 100x20mm culture plates was combined. 10μg of total RNA was fractionated by electrophoresis and transferred to nylon membrane as described in the methods section. Then, the RNA was hybridized with COLα1(I) cDNA probe. The northern blot analysis is a representative of two separate experiments. 18s rRNA represents the loading control.

Cyclic AMP Assay for Rat-1 Cells with Forskolin and PGE_2

The effects of forskolin and PGE_2 in Rat-1 cells were also examined with respect to cAMP production (Figure 16). PGE_2 (1μM) had no effect on cAMP levels when compared to the control levels. However, forskolin increased the cAMP levels by approximately 10-fold from the control level.

In summary, EP2 receptor expression was predominant in IMR-90 and only EP4 was detected in Rat-1 cells. In IMR-90, 11-deoxy PGE_1 mimicked the effects of PGE_2 on the three genes to include the cytokine induced effects on COLα1(I) and LO mRNA levels. Further, forskolin, an adenylyl cyclase activator, demonstrated very similar effects on the three genes as PGE_2 and 11-deoxy PGE_1. However, the effects of PGE_2 and forskolin on cAMP production in IMR-90 were different than in Rat-1 cells. Also, the maximal level of cAMP produced by PGE_2 was different from that of forskolin.

Figure 16


Effect of Forskolin and PGE_2
on cAMP Production in Rat-1 Cells
^{3}H-cAMP (cpm)
6000
5000
4000
3000
2000
1000
0
CONTROL
PGE_2
FORSKOLIN

Legend for Figure 16

Rat-1 cells were labeled with ^{3}H-adenine as described in the methods section. The cells were treated with IBMX and with or without (control) PGE_2 (1μM) or forskolin (25μM) for 30 minutes. ^{3}H-cAMP was eluted and quantitated as described in the methods section. Values are the means +/- standard error of three experiments.

DISCUSSION

The results illustrate that human embryonic lung fibroblasts, IMR-90, express EP2, EP3 and EP4 receptor mRNAs, but no detectable level of EP1 mRNA. Among these, the EP2 receptor mRNA is predominant while EP4 and EP3 receptor mRNAs are expressed to a lesser degree with EP3 showing the least amount. The receptor types were stimulated with agonists, sulprostone for EP3/EP1 receptors and 11-deoxy PGE_1 for EP2/EP4 receptors, to determine their functions with respect to PGE_2 inhibition/stimulation of COLα1(I), LO and COX-1 mRNA expressions. Sulprostone did not show any effect on the expression of the three genes of interest. On the other hand, 11-deoxy PGE_1 at 1μM decreased the basal levels of COLα1(I) and LO mRNA, and increased the COX-1 mRNA basal level. These results were comparable to the effects observed with PGE_2 at 1μM. Interestingly, 11-deoxy PGE_1 at a lower concentration increased the basal level of COLα1(I) mRNA, only. Additionally, 11-deoxy PGE_1 abrogated the TGF-β and IL-1β induced COLα1(I) and LO mRNA levels, respectively. These results strongly suggest that EP2 and possibly EP4 receptors are key mediators of PGE_2 in the regulation of COLα1(I), LO and COX-1 gene expressions in IMR-90 cells.

The EP2 [Regan et al., 1994] and EP4 [An et al., 1993; Bastien et al., 1994] receptors have been reported to increase cAMP. Therefore, we examined the effects of a known cAMP stimulator, forskolin, on COLα1(I), LO and COX-1 mRNA expressions. Forskolin not only limited the basal COLα1(I) and LO mRNA

levels, but increased the basal COX-1 mRNA levels. The effect was comparable to the effect of PGE_2. Interestingly, the co-incubation of forskolin and PGE_2 in IMR-90 limited COLα1(I) mRNA production to a greater degree. However, PGE_2 and forskolin together did not have any additive effects on LO or COX-1 mRNA levels.

It is possible that the synergy between PGE_2 and forskolin on COLα1(I) mRNA expression is due to the sub-optimal stimulation of cAMP by either agent alone. This hypothesis appears to be true according to results shown in Figure 10. In this figure, 1μM of PGE_2 or 25μM of forskolin produced sub-optimal levels of cAMP while their combined effects produced the maximal level. However, when cAMP levels were measured in response to increasing PGE_2 concentrations, 1μM of PGE_2 produced the maximal levels of cAMP as shown in Figure 11.

The comparison of the two results suggests that forskolin and PGE_2 may be inducing cAMP production and subsequent downstream effectors in different manner. This may be possible by targeting different isoforms of cAMP signaling components such as adenylate cyclase, cAMP PDE or PKA. To date, nine isoforms of adenylate cyclases and approximately 30 isoforms of cAMP PDE have been reported in mammalian cells [Houslay and Milligan, 1997].

Forskolin has been reported to activate all but one isoform of adenylate cyclases. The adenylate cyclase isoform(s) involved in EP2 or EP4 receptors signaling have not been determined yet. It is possible that the two receptors

interact with more limited number of adenylate cyclase isoforms, which could be co-located with the receptors, than forskolin. Further, it is possible that the cAMP signaling components (adenylate cyclase, cAMP PDE, PKA) that are specific for the EP receptors could be maintaining a certain threshold level of cAMP for the receptors' signaling mechanisms. Such a threshold level may be reason for EP receptors to produce less cAMP levels than forskolin.

In 1996, Jurevicius and Fischmeister reported the co-localization of phosphodiesterase with the cAMP signaling cascade of beta-adrenergic receptors in frog ventricular cells. Consequently, the intracellular cAMP level was not uniform throughout the cell whenever the receptor signaling cascade was activated. This suggests that the cAMP signaling components are not only specific but also spatially organized. Further, the additive effect of PGE_2 and forskolin in suppression of COLα1(I) mRNA level, shown in Figure 7, seems to indicate that inhibition of COLα1(I) gene expression could be induced by more than one type of cAMP signaling mechanism.

Recent publications indicate that EP4 and EP2 similarly bind at high affinity with PGE_1, PGE_2, 11-deoxy PGE_1 and 16, 16-dimethyl-PGE_2 [Kiriyama et al., 1997]. Therefore, it is possible that in IMR-90, both EP2 and EP4 receptors are active in the mechanism limiting the production and deposition of collagen. However, there are some important structural and functional differences between these two receptors. For example, in the cloned mouse EP4 receptors, there are about 3.5 fold more serine and threonine residues in the C-terminal than in the

mouse EP2 receptors [Nishigaki et al., 1996]. Also, EP4 receptor contains potential PKA phosphorylation sites whereas none has been found in EP2 receptor [Nishigaki et al., 1996]. Such phosphorylation sites may be critical in receptor desensitization [Liggett et al., 1993] and subsequently, the termination of signal transduction. Nishigaki et al (1996) demonstrated a difference in PGE_2 induced-desensitization between EP4 and EP2 receptors in CHO cells. In this experiment, PGE_2 induced EP4 receptors to undergo rapid short-term desensitization while it had no such effect on EP2 receptors. Importantly, EP2 receptors generated a higher adenylate cyclase activity than EP4 receptors a in time dependent manner. Also, EP2 receptor proved more responsive to 15-keto-PGE_2, a PGE_2 metabolite, than EP4 in stimulating adenylate cyclase. Interestingly, 15-keto-PGE_2 is metabolized by 15-hydroxy PG dehydrogenase which is an enzyme that is abundant in the lung [Anggard et al., 1971]. Hence, EP2 receptor mRNA being the predominately expressed gene, and more capable in producing cAMP than EP4 receptor, is most likely responsible for the inhibitory effect of PGE_2 on COLα1(I) and LO mRNA levels in IMR-90.

Rat-1 cells are active producers of the COLα1(I) peptide [Granot et al., 1993]. Examination of PGE_2 receptor types in these cells shows that they express EP4 mRNA but lack detectable EP1, EP2 and EP3 mRNA. These cells were treated with PGE_2 or forskolin to examine whether either effected COLα1(I) mRNA and cAMP levels. Surprisingly, forskolin increased the COLα1(I) mRNA levels. PGE_2 also had a different effect in Rat-1 cells than in IMR-90. PGE_2

slightly increased COLα1(I) mRNA level in Rat-1 cells. Furthermore, PGE_2 had no effect on cAMP levels with 30 min incubation, while forskolin increased it by 10-fold. Interestingly in the Rat-1 cells, EP4 receptors are predominant. These results further suggest that EP4 is a poor inducer of cAMP production which could not be detected after 30m incubation with PGE_2. These results strengthen the hypothesis that the EP2 receptors are the receptors mediating PGE_2 effects on the COLα1(I), LO and COX1 gene expression.

Further, 11-deoxy PGE_1, at low concentration (10nM) in IMR-90 (Figure 3) increased COLα1(I) mRNA level. One explanation for this is that at low concentrations, 11-deoxy PGE_1 could be more potent for EP4 receptor which may be linked to a mechanism involved in increasing the COLα1(I) mRNA level. In fact, the Ki of EP4 and EP2 for 11-deoxy PGE_1 is 23nM and 45nM, respectively, while Ki for PGE_2 is 1.9nM and 12nM, respectively [Kiriyama et al., 1997]. These lower Ki's could possibly cause the EP4 receptors become activated at low concentrations.

Although both EP2 and EP4 receptors stimulate cAMP synthesis, they may effect different isoforms of cAMP signaling components such as PKA isoforms, thus eliciting different cellular responses. The type II regulatory subunit (RII) of PKA is widely known to be associated with an A-kinase anchoring protein, AKAP, and is insoluble [Johnson et al., 1997; Visconti et al., 1997; Feliciello et al., 1997; Pawson and Scott, 1997; Dell'Acqua and Scott, 1997]. According to Visconti et al. (1997), dibutyryl cAMP, a cAMP analog, did not

dissociate the catalytic subunit associated with the RII in cauda epididymal mouse sperm. If EP2 receptor generated cAMP targets RII, this may explain why dibutyryl cAMP did not induce any decrease in COLα1(I) mRNA level, reported by Fine et al. (1992).

In addition, previous studies using MC3T3-E1, a mouse osteoblast-like cell line, showed that forskolin and other agents which increase cAMP levels increase COL synthesis [Hakeda et al., 1987]. On the other hand, an elevation of cAMP in UMR 106-06 rat osteosarcoma cells was shown to decrease collagen synthesis [Iida-Klein et al., 1992; Pun 1989]. The understanding of these apparently contradictory effects of cAMP clearly warrants further investigation and may ultimately prove important in the role of PGE_2 as a regulator of collagen synthesis and the fibrotic response.

It appears that limitation by PGE_2 of collagen production and its deposition as an extracellular matrix involves the EP2 receptor with cAMP at the core of this signal transducing mechanism. These results may be pertinent to mechanisms related to tissue scarring or connective tissue-linked diseases. In fact, the limiting by PGE_2 of cytokine stimulated collagen deposition and the failure or reversal of this mechanism could prove critical to the fibrotic sequel of inflammation and in certain connective tissue diseases.

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